DISSERTATION

# CHARACTERIZATION OF MEDIATORS OF CARDIAC AND RENAL DEVELOPMENT IN RESPONSE TO INCREASED PRENATAL TESTOSTERONE

Submitted by

Ryan W. Maresh

Department of Biomedical Sciences

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY RYAN W. MARESH ENTITLED CHARACTERIZATION OF MEDIATORS OF CARDIAC AND RENAL DEVELOPMENT IN RESPONSE TO INCREASED PRENATAL TESTOSTERONE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

_____
Dr. James Barry

_____
Dr. Richard Bowen

_____
Dr. Charles Miller

_____
Dr. Christopher Orton

_____
Advisor: Dr. Russell Anthony

_____
Department Head: Dr. Barbara Sandborn

ABSTRACT OF DISSERTATION

CHARACTERIZATION OF MEDIATORS OF CARDIAC AND RENAL DEVELOPMENT IN RESPONSE TO INCREASED PRENATAL TESTOSTERONE

During fetal development, a complex interaction between numerous growth factors, receptors, and signaling pathways takes place to establish the regulation of the various hormonal and metabolic functions necessary for normal development, both during the fetal period as well as later in life. Alterations in the fetal environment during this time can modify normal gene expression, protein concentrations, metabolic cascades, and other physiologic functions. Exposure to excess prenatal androgens has been previously shown to cause growth restriction, alter sexual development and function, and cause the onset of symptoms that closely resemble those seen in women with polycystic ovary syndrome, including the metabolic syndrome.

Previously reported differences in cardiac and kidney weights at 21 months of age of female offspring from ewes treated with testosterone during early- to mid-gestation suggested the development of systemic hypertension. This observation led to the current study to evaluate if prenatal androgen excess influences cardiovascular development and can lead to adulthood disease by altering the expression of key mediators in the heart and kidneys, as well as if it can alter metabolic mediators important in glucose regulation.

Pregnant Suffolk ewes were assigned to either a control or a prenatal testosterone

treatment group. The treated ewes received twice weekly im injections of 100 mg testosterone (T)-propionate in 2.4 ml cottonseed oil from days 30 – 90 of gestation (term = 147). The control ewes received im injections of vehicle only. Lambs were weaned at 8 weeks of age and a sub-set of control and growth restricted prenatally T-treated females were maintained until either 9 months (con = 6; treat = 5) or 21 months (con = 8; treat = 8) of age. In addition, female fetuses at 65 dGA (con = 8; treat = 9) and 90 dGA (con = 8; treat = 8) were collected. Heart and kidney tissues were collected at each end point, weighed, frozen in liquid nitrogen, and stored at -80°C for later analysis.

Total cellular RNA and protein were isolated from fetal total hearts, fetal kidney, adult left ventricle, adult right ventricle, and adult kidney samples. Real-time PCR and Western immunoblot analysis were performed for angiopoietin 1 (Ang1), angiopoietin 2 (Ang2), their receptor tunica interna endothelial cell kinase-2 (Tie2), angiotensin II receptor subtypes 1 and 2 (AT1 and AT2), endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), VEGF receptor 1 (VEGF R1), VEGF receptor 2 (VEGF R2), insulin receptor β (IRβ), glucose transporter 1 (GLUT1), and glucose transporter 4 (GLUT4). We hypothesized that prenatal exposure to excess testosterone from days 30 to 90 of gestation will up-regulate the mRNA and protein concentrations of angiogenic growth factors and mediators of insulin signaling and glucose metabolism in treated fetal cardiac and kidney tissues and then decrease postnatally in an age-dependent manner.

No changes were detected in any of the mediators at fetal Day 65. While no changes were detected in the left ventricle at any age for AT1 mRNA, a significant decrease ($p = 0.004$) was seen in the right ventricle of the treatment group at 21 months

of age. In contrast to AT1, by fetal Day 90, changes in cardiac AT2 mRNA concentration tended to be reduced ($p = 0.102$) in the treatment group but this difference disappeared with age. Concentrations of VEGF and VEGF R1 mRNA in the heart were not impacted by prenatal T-treatment at any age examined, although VEGF R2 mRNA concentration tended ($p = 0.059$) to be elevated in the left ventricle of T-treated offspring at 9 months of age. At 21 months of age, Ang1 mRNA concentration in the treatment group was significantly reduced ($p = 0.025$) in the left ventricle and was also decreased ($p = 0.098$) in the right ventricle. No cardiac changes were detected in Ang2 or Tie2 mRNA or protein concentrations at any age. In the left ventricle, eNOS mRNA concentration tended to be elevated in the treatment group ($p = 0.084$) at 9 months of age, but by 21 months of age, tended to be reduced ($p = 0.052$) in the treatment group. In the right ventricle, eNOS mRNA concentration was significantly reduced ($p = 0.019$) in the treatment group at 9 months of age and even more significantly reduced ($p < 0.001$) by 21 months of age. Protein data also showed a significant decrease ($p = 0.032$) in eNOS in the left ventricle at 21 months of age. Interestingly, protein data for eNOS in the right ventricle indicated an increase ($p = 0.052$) in the treatment group at 21 months of age, suggesting that there may be effects within the pulmonary circulation that are differentially impacting the right side of the heart.

In the kidney, no significant differences were seen in any of the angiogenic/vasculogenic mediators at fetal Day 65. In addition, no changes in renal Ang1, Ang2, Tie2, AT1, AT2, or VEGF R1 mRNA or protein concentrations were detected at any time point. At fetal Day 90, VEGF mRNA concentration was significantly lower ($p = 0.044$) in the treatment group compared to controls. This

significance disappeared by 9 months and by 21 months of age, a significant decrease ($p = 0.024$) in VEGF R2 mRNA concentration in the treatment group was found. At fetal Day 90, eNOS mRNA concentration tended to be increased ($p = 0.059$) in the treatment group, and while still elevated, the significance was lost by 9 months of age. In adult renal tissues, the eNOS concentration at 9 months of age was significantly elevated ($p = 0.027$) in response to prenatal T-treatment, but by 21 months of age the difference was lost. The age differential in eNOS between the heart and kidney indicates that a shift in vasoregulation is occurring. This shift within the kidney, starting at fetal Day 90, may be driving the changes that are seen in the other mediators at 21 months of age. Taken together, these changes suggest that the kidney is beginning to fail to maintain renal tissue function and survival due to the loss of VEGF signaling. When taken as a whole, the data supports the development of systemic hypertension as offspring exposed to prenatal androgen excess age.

As with the angiogenic/vasculogenic and hemodynamic mediators, few changes were detected in the cardiac metabolic mediators evaluated. At fetal Day 90, a significant increase ($p = 0.021$) in the mRNA concentration of IRβ was seen in the cardiac tissue from prenatally T-treated fetuses. At 9 months of age, left ventricle IRβ mRNA concentration was no longer elevated in the treatment group despite the presence of a significant increase ($p = 0.009$) in IRβ concentration. Interestingly, a shift in IRβ concentration was seen in the left ventricle at 21 months of age, where it was significantly reduced ($p = 0.036$). At 9 months of age, GLUT4 mRNA concentration tended to be reduced ($p = 0.097$) in the treatment group and this decrease was even more significant ($p = 0.003$) in the right ventricle at 21 months of age, suggesting that the right

ventricle is becoming insulin resistant with age. By 21 months of age, significant decreases in GLUT1 mRNA concentration were detected in both the left ($p = 0.013$) and right ventricles ($p = 0.006$).

Few changes were detected in the renal metabolic mediators evaluated at any of the time points studied. The only significant change occurred at 9 months of age where a significant increase ($p = 0.047$) in GLUT4 mRNA concentration was seen in the treatment group. No differences were found in the protein data at 9 and 21 months for the metabolic mediators evaluated in renal samples.

Taken as a whole, the data supports a shift in vasoregulation and the development of insulin resistance as offspring exposed to excess prenatal androgen age. The lack of many early changes in the cardiovascular and metabolic mediators suggests that there is less of a direct effect of excess androgen on fetal cardiovascular development and metabolic regulation and more of an indirect effect on other aspects of the uterine environment, such as placental development, that have long-term effects that manifest during adulthood. The characterization of mediators of vascular and metabolic development in fetal and adult tissues in the current study helps to more thoroughly understand the effects of excess prenatal androgen on fetal development and subsequent adult disease states.

Ryan W. Maresh
Department of Biomedical Sciences
Colorado State University
Fort Collins, Colorado 80523
Summer 2008

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

# CHAPTER I

## INTRODUCTION

With any pathological condition, a common question arises: Is the cause of the disease genetic, due to the environment we have lived in, or simply by chance? Many times it is difficult to establish a definitive answer because often all are involved. While the genetic component plays a significant role for increasing susceptibility to some diseases, the environment in which those genes exist may also alter how, when, or if, they are expressed. In the nature vs. nurture debate, there are few, if any, clear answers. In some cases, genetics are unquestionably the cause, such as with Tay-Sachs disease or Down syndrome. In others, the impact of the environment is obvious and dramatic—the temperature of the egg during incubation determines whether the American alligator becomes a male or a female. While the environment does not determine the sex of human embryos, there is a large and growing body of evidence that supports its role in influencing embryonic and fetal development, and that that influence is important in the development and onset of chronic diseases later in adult life.

There is no question that lifestyle choices increase our susceptibility to developing cardiovascular disease and diabetes. However, it has been well documented that the *in utero* environment plays a critical role in programming fetal anatomic and physiologic development, and that these changes persist into postnatal life. During fetal development, tissues and organs undergo critical periods of development that coincide

with periods of rapid cell division (Barker DJP 2001). Disturbances during these critical periods can influence homeostatic mechanisms, the effects of which may be subtle during development, but have long-lasting negative impacts on adult health (Hoet and Hanson 2001). In growth restricted fetuses, short-term adaptations are beneficial for fetal survival but are often detrimental to health later in life. These adaptations can permanently alter tissue, as well as whole body structure, function, and metabolism. Adulthood diseases linked to fetal growth restriction (FGR) and fetal programming include cardiovascular disease, hypertension, type II diabetes, obesity, and various reproductive disorders.

Among women, polycystic ovary syndrome (PCOS) is a common endocrine disorder, affecting 5 – 10% of pre-menopausal women. Women with PCOS often display signs of the metabolic-linked disease, such as diabetes and hypertension. In addition, mothers with PCOS have been shown to have a significantly higher incidence of small for gestational age infants. Pregnant women with PCOS display a significant increase in circulating androgens, which may readily pass to the fetus. Monkey and sheep models of prenatal androgen excess also display symptoms of PCOS, and sheep also exhibit fetal growth restriction. Similarities between the prenatal androgen excess sheep model and women with PCOS suggest common metabolic mediators between androgen-induced FGR and fetal programming that lead to the metabolic disturbances later in life. We hypothesized that prenatal androgen excess influences cardiovascular and metabolic development and leads to adulthood disease by altering mRNA and protein concentrations in the heart and kidneys.

Using an ovine model of polycystic ovary syndrome induced by prenatal androgen excess leading to fetal growth restriction, I characterized the time-course concentration of cardiac and metabolic markers of cardiovascular development and glucose metabolism that may contribute to the onset of the adulthood diseases hypertension and type II diabetes. I hypothesized that prenatal exposure to excess testosterone from days 30 to 90 of gestation will up-regulate the mRNA and protein concentrations of angiogenic growth factors and mediators of insulin signaling and glucose metabolism in treated fetal cardiac and kidney tissues and then decrease postnatally in an age-dependent manner.

# CHAPTER II

## REVIEW OF LITERATURE

The prevalence and economic costs of cardiovascular disease and diabetes in the United States has increased drastically over the last decade. Cardiovascular disease, including hypertension, coronary heart disease, and stroke, accounts for more than 930,000 deaths each year, while diabetes contributes over 71,000 deaths (Eyre *et al.* 2004). Combined, total direct and indirect costs of cardiovascular disease and diabetes amount to almost $500 billion annually (Eyre *et al.* 2004; Thom *et al.* 2006). While there are a number of life-style factors that contribute to the development of these diseases, there is strong, and growing evidence that the fetal environment plays a critical role in programming fetal anatomic and physiologic development, and that these changes persist into postnatal life increasing the susceptibility to such adulthood diseases.

The fetal origins of adult disease became more widely acknowledged following the work of Dr. David Barker and his colleagues and states that environmental factors act during prenatal development to program the risks for the onset of cardiovascular and metabolic diseases in adult life and can ultimately lead to premature death (Mcmillen and Robinson 2005). Fetal programming describes the process where a stimulus or insult during a critical, sensitive period of development has lifelong effects by altering metabolism and physiology (Barker 2000; Xita and Tsatsoulis 2006; Lucas 2005). Any number of environmental stressors can potentially have negative consequences on fetal

development, such as hypoxia, maternal nutrition, hyperthermia, reduced placental blood flow, and stress. Normal fetal growth requires that the mother's capacity to supply nutrients and the placenta's capacity to transport them to the fetus meet the demands of the growing fetus (Van Assche *et al.* 1998). In addition, the fetal genome is actively producing fetal growth factors, both stimulatory and inhibitory, that regulate fetal growth and development. For normal fetal growth and development to occur, homeostasis between the maternal, placental, and fetal components must occur. An imbalance in any of these can lead to changes in gene expression that can alter fetal growth, development, and survival. As a result of sub-optimal fetal environment, overall fetal growth is restricted in an attempt to protect the critical organs.

## Fetal Growth Restriction

As the connection between suboptimal fetal environment, fetal growth restriction, and adult disease continues to grow, it is increasingly important that fetuses and babies that are small at birth be correctly identified. Often, the terms fetal growth restriction, intrauterine growth restriction, and small for gestational age (SGA) are used interchangeably. While fetal growth restriction and intrauterine growth restriction can be, they are different from small for gestational age. One of the problems with accurate assessment of a small baby is with the population of infants that should be included to define the standard (Goldenberg and Cliver 1997). However, despite the confusion, a consensus has been reached for defining and differentiating between fetal growth restriction and small for gestational age.

Fetal growth restriction occurs when the fetal growth rate is decreased to the point that an infant does not reach its full genetic growth potential at a given gestational age

(Mamelle *et al.* 2001; Anthony *et al.* 2003; Brodsky and Christou 2004; Lin and Santolaya-Forgas 1998; Pollack and Divon 1992) and applies to births where the offspring weigh less than the 10$^{th}$ percentile of birth weight-for-gestational age (Goldenberg and Culhane 2007). Fetal growth restriction differs from preterm birth which is defined as birth that occurs earlier than 37 weeks from the first day of the last menstrual cycle, regardless of birth weight (Goldenberg and Culhane 2007). It is also differentiated from small for gestational age, which refers to an infant of abnormal size, either in weight and/or length, who is below the 10$^{th}$ percentile for that gestational age at birth (Mamelle 2006). The majority, up to 70%, of small for gestational age infants are due simply to constitutional factors that are determined by maternal ethnicity, parity, weight, or height (Lin and Santolaya-Forgas 1998). Fetal growth restricted infants are classified as small for gestational age if their birth weight is less than 3 – 10% based on standard growth curves, and make up only a small percentage of SGA infants (Brodsky and Christou 2004; Lin and Santolaya-Forgas 1998). In growth restricted fetuses, the short-term adaptations are in response to a pathological condition and are beneficial for fetal survival but are often detrimental to health later in life. This negative outcome in adulthood may result from a later insult that triggers a self-perpetuating cycle of progressive loss of function (Barker *et al.* 2002; Brenner and Chertow GM 1994).

Fetal growth restriction varies depending on the type of insult, the stage of gestation, and the duration (Lin and Santolaya-Forgas 1998). It typically occurs in two forms: symmetric (20 – 30%) and asymmetric (70 – 80%) intrauterine growth restriction and is affected by the timing of the maternal insult (Brodsky and Christou 2004). Fetal growth that is restricted during early to mid gestation (first or second trimester) typically

results in symmetric growth restriction and occurs due to a proportional decrease in overall growth that results from a reduction in cellular proliferation of all organs (Brodsky and Christou 2004; Lin and Santolaya-Forgas 1998). Most often, however, fetal growth restriction results in asymmetric growth where abdominal size is decreased relative to head size (Brodsky and Christou 2004; Anthony *et al.* 2003). It occurs due to a decrease in growth velocity during the last trimester, most often due to functional placental insufficiency, that results in impaired fetal cellular hypertrophy in less critical tissues in order to protect the brain (Brodsky and Christou 2004). This "head-sparing" effect demonstrates the ability of the fetus to adapt to a suboptimal environment and redistribute cardiac output. Other maternal factors, such as vascular diseases due to hypertension and diabetes mellitus, can also lead to fetal growth restriction (Lin and Santolaya-Forgas 1998).

Fetal growth restriction continues to cause infant mortality and morbidity that affects up to 8% of pregnancies in developed countries and 17% in developing countries (Wallace *et al.* 2005). It is also a major risk factor for the development of cardiovascular diseases, hypertension, type II diabetes, dyslipidemia, and obesity, the combination of which is more commonly referred to as the metabolic syndrome, and various reproductive disorders (Wallace *et al.* 2005; Barker DJP 1998; Barker *et al.* 2002; Manikkam *et al.* 2004). Even if an infant is not considered to have low birth weight, alterations in endocrine function can occur, as well as the programming of hypertension, later in adulthood (Wintour *et al.* 2003; Fowden *et al.* 2005). As such, it is critical that the fetal environment and its impact on fetal development, and ultimately adulthood

health, be better characterized. To this end, several models of fetal growth restriction exist to study the development and progression of growth restriction.

**Animal Models of Growth Restriction and Androgen Excess**

Fetal growth restriction in the pregnant sheep has been induced using numerous methods: nutrient restriction or excess, administration of glucocorticoids, uterine artery ligation, caruncle ctomy to limit placental implantation sites, utero-placental embolization, and exposure to increased ambient temperatures (Anthony *et al.* 2003). In addition, Manikkam *et al.* have shown that an ovine model of polycystic ovary syndrome (PCOS) leads to fetal growth restriction (Manikkam *et al.* 2004) and a follow-on evaluation of female offspring at 21 months of age indicated increased cardiac and kidney weights in the prenatal testosterone treated ewes, suggesting the development of systemic hypertension (Padmanabhan *et al.* 2004). Using the same model, King *et al.* have shown that exposure to prenatal androgen excess leads to mild hypertension in 2 year old adult ewes (King *et al.* 2007), and others have demonstrated impaired insulin sensitivity in female offspring starting during early postnatal life and at 18 months of age (Recabarren *et al.* 2005; Kavoussi *et al.* 2006). This model has clinical relevance as women with polycystic ovary syndrome, in addition to the endocrine effects, also develop signs of the metabolic syndrome and have a higher incidence of fetal growth restricted offspring. Until recently, this model, in both rhesus monkeys and sheep, has focused primarily on the hypothalamic-pituitary-gonadal axis during prepubertal development and adult reproductive life (Recabarren *et al.* 2005; King *et al.* 2007).

The nonhuman primate model of PCOS utilizing the female rhesus monkey has proven to be very appropriate for understanding the outcomes of prenatal androgen

excess. In this model, exposure of female fetuses to testosterone levels equivalent to those found in fetal males develop the clinical and biochemical features later as adults that are found in PCOS women (Abbott *et al.* 2005). Metabolic defects common between the rhesus monkey model and women with PCOS include aberrant adipose tissue distribution, with a preferential disposition of visceral fat as adults that is independent of obesity (Eisner *et al.* 2003), defects in insulin secretion and action, as well as altered insulin-glucose homeostasis (Eisner *et al.* 2000). The effect of androgen excess on insulin and glucose metabolism depends on the timing of the exposure and the critical period of development that the fetus is in. Exposure to excess androgen during early fetal development tends to impair insulin secretion (Eisner *et al.* 2000), indicating that androgens may alter the developing pancreas and β-cell function. Late gestation exposure decreases insulin sensitivity with increasing adiposity (Eisner *et al.* 2000). This suggests that late exposure is beyond the critical period of pancreatic and β-cell development, therefore secretion is not impacted, but the androgens can affect the target tissues of insulin. The timing of exposure is also important in affecting the organization and development of the neural network, especially the GnRH pathway. In the rhesus monkey and humans, exposure during the first trimester results in altered sexual differentiation of the GnRH network (Robinson 2006; Herman *et al.* 2000) and in the sheep this critical period of effect is around 30 – 90 days of gestation (Robinson 2006; Clarke *et al.* 1976; Wood *et al.* 1995). This differential timing of fetal androgen excess may help to account for the heterogeneity of the PCOS phenotype expressed by women and be important for the expression of PCOS traits that are outside of the standard diagnostic criteria (Xita and Tsatsoulis 2006).

Recent studies have begun to evaluate the metabolic effects of glucose metabolism and insulin resistance (Eisner *et al.* 2000; Recabarren *et al.* 2005) using glucose tolerance tests and insulin sensitivity indices, but little attention has been given to the cellular mechanisms involved in the development of the metabolic syndrome commonly found in PCOS. As previously discussed, rhesus monkey and sheep models of prenatal androgen excess display symptoms of PCOS, and sheep also exhibit fetal growth restriction (Manikkam *et al.* 2004; Recabarren *et al.* 2005). Of these, the sheep model provides several advantages that allow relevant comparisons to humans. These advantages include the ability to study singleton pregnancies, comparable birth weights, similar organogenesis for major organ systems, equivalent rates of fetal protein accretion and a similar ratio of maternal:fetal body weight (Wallace *et al.* 2005). Overall, similarities between the sheep model of prenatal androgen excess and women with PCOS suggest common metabolic mediators between androgen-induced FGR and fetal programming that lead to the metabolic disturbances later in life.

## Polycystic Ovary Syndrome

Polycystic ovary syndrome is an endocrine disorder affecting 5 – 10% of reproductive age women and displays a wide phenotypic heterogeneity (Sir-Petermann *et al.* 2002b; Sir-Petermann *et al.* 2002a; Xita *et al.* 2002). It is important to distinguish the disorder as a syndrome, rather than a disease, due to the complex of symptoms displayed by affected women. While the etiology and pathogenesis of PCOS is still uncertain, the syndrome appears to be a familial disorder with a strong genetic basis (Franks 2002; Franks 1995; Abbott *et al.* 2002; Xita *et al.* 2002). However, the mode of inheritance is unclear. Some studies have reported an autosomal dominant pattern of inheritance

(Ferriman 1979; Carey 1993) while others have found that this mode of inheritance can not be explained (Hague 1988). Still others have suggested an X-linked mode of inheritance (Givens 1988). The differences in the mode of inheritance, and the range of symptoms, indicate that PCOS is a multi-gene disorder that is differentially inherited and this fact may help to explain the various clinical and biochemical phenotypes. There also appears to be significant ethnic and racial variations in the clinical presentation of PCOS, the frequency of obesity, insulin resistance, and incidence of diabetes mellitus (Xita *et al.* 2002). A number of genes have been investigated for not only their involvement in the cause of PCOS, but also how they are affected by the onset of the disorder. These genes include those involved in reproduction, those that affect the secretion or action of insulin, those coding for steroidogenic enzymes in the androgen biosynthetic pathway, and those involved in obesity and energy production (Xita *et al.* 2002; Franks *et al.* 2006). In addition to the genetic aspects of the syndrome, it appears that the phenotypic expression of PCOS is also influenced by environmental factors. Franks *et al.* have summarized the interaction in their hypothesis that "PCOS is a genetically determined ovarian disorder characterized by excessive androgen production and that the heterogeneity can be explained on the basis of the interaction of this disorder with other genes and the environment" (Abbott *et al.* 2002; Franks *et al.* 2006).

As mentioned above, women with PCOS display a wide heterogeneity in phenotype, with a spectrum of endocrine and biochemical symptoms. Clinical manifestation of PCOS includes: polycystic ovaries, irregular menses (oligomenorrhea or amenorrhea), chronic anovulation, infertility, hyperandrogenism, hirsutism, obesity, and reduced β-cell function (Sir-Petermann *et al.* 2005; Xita *et al.* 2002; Franks *et al.*

2006). However, there are women with polycystic ovaries that are endocrinologically normal (Xita *et al.* 2002). In addition, there is strong support for an association between PCOS and long-term disease risks. Women with PCOS often display signs of the metabolic syndrome, such as hyperinsulinemia, type II diabetes, peripheral insulin resistance, cardiovascular disease, hypertension, and dyslipidemia (Sir-Petermann *et al.* 2002a; Sir-Petermann *et al.* 2005; Orio *et al.* 2006). Up to half of all PCOS women meet the criteria for the metabolic syndrome, with insulin resistance implicated as the pathological link between the two syndromes (Essah *et al.* 2007). Insulin resistance causes a state of low-grade, chronic systemic inflammation and links the metabolic and vascular pathologies (Bansilal *et al.* 2007). In addition, insulin resistance leads to increased lipolysis in fat cells and the release of free fatty acids, resulting in dyslipidemia and oxidative stress (Bansilal *et al.* 2007). The combination of dyslipidemia and oxidative stress then leads to endothelial dysfunction and vascular damage, with over 90% of all women with PCOS having at least one adverse cardiovascular risk factor (Essah *et al.* 2007; Cersosimo and DeFronzo 2006). Most women with PCOS, regardless of weight, experience some degree of insulin resistance with compensatory hyperinsulinemia (Dunaif *et al.* 1989). In fact, PCOS women have a seven times greater prevalence of glucose intolerance and type II diabetes than normal women (Franks 1995). Insulin resistance and type II diabetes in PCOS women is independent of obesity and is present in non-obese PCOS women (Nestler and Jakubowicz 1997). As in non-PCOS obese women, however, obesity increases the risk and severity. For obese women with PCOS, insulin resistance is reversible with weight loss, despite the fact that elevated levels of insulin secretion from pancreatic β-cells still exists (Holte *et al.* 1995). In the

case of PCOS, it is thought that the hyperinsulinemia may play a role in the pathogenesis of hyperandrogenism (Nestler *et al.* 1989). In adolescent females who were born small, both insulin and free androgen levels have been independently associated with central fat mass, indicating that hyperinsulinemia and hyperandrogenism may promote an android distribution of body fat (Ibanez *et al.* 2003). While the role of hyperandrogenism in the pathogenesis of central adiposity in PCOS women remains controversial, there is an association with visceral fat accumulation and elevated androgens in females (Xita and Tsatsoulis 2006). This connection is present even though there is evidence that testosterone affects lipolysis to decrease visceral fat storage (Wajchenberg 2000).

**Polycystic Ovary Syndrome and Programming**

The clinical manifestation of PCOS during adolescence occurs along with the maturation of the hypothalamic-pituitary-ovarian axis (Franks 2002). While the development of PCOS symptoms typically does not occur until the onset of puberty, the underlying causes are most likely present since birth, or earlier. The growing body of evidence in both rhesus monkey and sheep models of PCOS supports the hypothesis that the origin of the syndrome is due to fetal programming. With the onset of puberty, changes in the hypothalamic-pituitary-ovarian axis, metabolic changes, and fat disposition may unmask the endocrine features of PCOS (Franks *et al.* 2006). In sheep, prenatal androgen excess results in a decreased sensitivity of the gonadotropin-releasing hormone (GnRH) neural network to negative feedback from the steroid hormones (Xita and Tsatsoulis 2006), as well as an increase in luteinizing hormone (LH) secretion (Robinson *et al.* 1999; Xita and Tsatsoulis 2006). The decreased sensitivity may be due to the signaling actions of the androgen receptor, which has been reported in the

hypothalamus (Manolagas and Kousteni 2001), rather than direct genomic effects. In fact, Masek *et al.* have reported that the increase in LH during puberty seems to be due to the effects of testosterone working through the androgen receptor (Masek *et al.* 1999). In PCOS women, 40% hypersecrete LH (Xita *et al.* 2002). Increased LH secretion can then lead to hyperandrogenism (Xita and Tsatsoulis 2006). The excess androgen exposure during the critical period of neural development results in an androgen-induced reprogramming such that the GnRH neurons are desensitized to normal steroid feedback (Xita and Tsatsoulis 2006). However, since most GnRH neurons lack androgen receptors, it appears that the activity of steroid sensitive inputs to the GnRH neurons is altered (Herbison 1995; Robinson 2006; Skinner *et al.* 2001). One possible explanation to this is that the desensitization occurs due to androgen alterations in the γ-aminobutyric acid secretion system, rather than to changes in the migration of the neurons during development (Sullivan and Moenter 2004). The precise mechanism that leads to reprogramming of the neuroendocrine axis in response to excess androgen exposure remains unclear. Since the neural network continues to develop in postnatal life, androgens potentially can continue to impact the organization and function for much longer periods (Robinson 2006). In addition to the reproductive aspects of PCOS, these changes may be the insult, or trigger, that leads to the onset of the metabolic syndrome seen in PCOS women, as it is possible that androgens have separate, but potentially cumulative, effects at the tissues (Robinson 2006).

## Pregnancy and Fetal Androgen Exposure

Pregnancy brings additional risks and effects to both mothers with PCOS and the developing fetus. In addition to the risk factors stated above, risk factors for pregnant PCOS women include gestational diabetes and pregnancy-induced hypertension (Xita *et al.* 2002). In addition to being hyperandrogenemic during pregnancy, mothers with PCOS have been shown to have a significantly higher incidence of small for gestational age infants (Sir-Petermann *et al.* 2005; Sir-Petermann *et al.* 2002b). The exposure of the fetus to elevated androgens during development is thought to be the cause of the low birth weight (Xita and Tsatsoulis 2006). This low birth weight in females has been related to the development of premature pubarche followed by the development of the metabolic syndrome (functional hyperandrogenism, insulin resistance with hyperinsulinemia, and dyslipidemia) during adolescence (Ibanez *et al.* 1998; Ibanez *et al.* 1996).

In a normal, healthy pregnancy, the female fetus is protected from maternal hyperandrogenism by the relatively high levels of maternal sex hormone-binding globulin and placental androgen metabolism (Abbott *et al.* 2002). Androgens, whether maternal or fetal in origin, are rapidly converted to estrogen by placental aromatase. However, if placental function is compromised, the possibility exists for the fetus to be exposed to elevated androgen levels. During pregnancy, women with PCOS display a significant increase in circulating androgens, which may readily pass to the fetus. Mean concentrations of maternal testosterone have been reported to be between 50 to 150% higher in PCOS women than in non-PCOS women, with large variations occurring between women (Franks 1995; Franks 1989; Conway GS *et al.* 1989; Yen SSC *et al.*

1970; Xita and Tsatsoulis 2006). The source of this excess androgen remains unclear, with possibilities including maternal sources, placental production, or fetal production. Maternal thecal cells and the placenta have been shown to increase androgen synthesis in response to hCG (Xita and Tsatsoulis 2006). While the fetal ovary is thought to not actively produce sex steroids during development, genetic or environmental factors could potentially stimulate steroidogenesis during fetal development, possibly in response to hCG, or later in early perinatal life due to gonadotropin release (Xita and Tsatsoulis 2006; Barbieri *et al.* 1986; Beck-Peccoz *et al.* 1991). Maternal hyperinsulinemia may stimulate excessive placental hCG secretion that leads to the development of fetal ovarian hyperplasia and hyperandrogenism (Xita and Tsatsoulis 2006; Barbieri *et al.* 1986).

The elevated maternal androgen levels are due to both a decrease in the concentration of sex hormone-binding globulin (SHBG) and an increased rate of androgen production. There is evidence that SHBG production is mediated by insulin, which suppresses the production of sex hormone-binding globulin (Anderson DC 1974; Franks 1989; Rosner 1990; Rajkhowa *et al.* 1994). In PCOS women, as well as in other individuals who are at risk for diabetes and cardiovascular disease, hyperinsulinemia increases the availability of circulating androgens by decreasing sex hormone-binding globulin (Rajkhowa *et al.* 1994; Lapidus *et al.* 1986; Lindstedt *et al.* 1991). During puberty, insulin levels rise and there is a subsequent fall in SHBG plasma levels (Toscano *et al.* 1992; Holly *et al.* 1989). This suggested that insulin, rather than sex hormones, may be more important in regulating SHBG levels. Nestler *et al.* showed that by inhibiting insulin secretion with diazoxide treatment, while suppressing ovarian steroidogenesis, a significant increase was seen in SHBG with no change in serum sex

steroid levels (Nestler *et al.* 1991). This indicates that hyperinsulinemia can decrease SHBG independently of the effects of sex steroids, at least in obese PCOS women (Nestler *et al.* 1991). They also provide support to the idea that insulin resistance and hyperinsulinemia in PCOS women is not caused by hyperandrogenism, but rather can lead to hyperandrogenism by insulin's stimulatory effect on ovarian androgen production and direct suppression of SHBG levels (Nestler *et al.* 1991).

In addition to insulin's impact on decreasing SHBG concentrations, there is also evidence of a genetic component in women with PCOS that results in decreased SHBG levels. Alterations in the *SHBG* gene in PCOS women have been related to decreased SHBG in several PCOS populations (Xita and Tsatsoulis 2006; Xita *et al.* 2003; Cousin *et al.* 2004). While the decrease in SHBG has potential consequences throughout life, low levels during fetal development may have a more significant effect as fetal tissues differentiate and develop.

Other than mediating SHBG, insulin has also been shown to influence androgen levels by altering the conversion of testosterone. Nestler has reported that insulin inhibits placental aromatase activity (Nestler JE and Strauss J III 1991; Nestler JE 1990; Nestler JE 1989; Nestler JE 1987). In placental cytotrophoblasts incubated with insulin, there was a 22 – 42% decrease in the aromatization of androgen compared to control cells (Nestler JE 1987). Aromatase activity was found to be 23 – 30% less in insulin-treated cells than in controls. Cytotrophoblasts that had been pretreated with insulin for 24 hours, washed, and incubated with fresh medium supplemented only with androstenedione also demonstrated a 27 – 35% reduction in androgen aromatization compared to controls (Nestler JE 1987). They determined that the deceased

aromatization was specifically due to a decrease in estrogen synthesis rather than increased estrogen catabolism (Nestler JE 1987). Nestler has also demonstrated that the action of insulin in placental cytotrophoblasts is selective and can be stimulatory. In cytotrophoblasts incubated with insulin, Nestler showed that 3β-hydroxysteroid dehydrogenase (3βHSD) activity is stimulated, potentially affecting placental steroidogenesis (Nestler JE 1989). In pregnant PCOS women with hyperinsulinemia, this reduced placental aromatase activity and increased 3βHSD activity potentially exposes the fetus to higher androgen levels than normal.

**Androgen Synthesis, Regulation, and Signaling**

There is evidence that PCOS may be the result of inappropriate activity of the enzyme involved in androgen synthesis (Xita *et al.* 2002). In obese women with PCOS, there is increased activity of ovarian P450c17α, a key enzyme in the biosynthesis of androgens (Sir-Petermann *et al.* 2002b; Nestler JE 1989; Nestler JE 1990). Abnormalities in P450c17α regulation typically result in an increase in 17α-hydroxylase, and to a lesser extent 17,20-lyase, activity of the enzyme (Rosenfield *et al.* 1994). Serine phosphorylation has also been implicated in the post-translational regulation of the 17,20-lyase activity (Xita *et al.* 2002; Zhang *et al.* 1995). The results of this increased enzyme activity are an increase in the conversion of cholesterol to pregnenolone, the initial step in steroidogenesis, and increased androgen secretion.

However, 20 – 50% of women with PCOS are not obese, but are normal weight or even thin. These women are still insulin resistant and hyperinsulinemic compared to non-PCOS women and exhibit increased P450c17α activity (Nestler and Jakubowicz 1997). It appears then, at least in PCOS, that P450c17α is stimulated by insulin. By reducing

insulin levels with the use of metformin in lean PCOS women, Nestler demonstrated a reduction in ovarian P450c17α activity, total testosterone, free testosterone, and androstenedione (Nestler and Jakubowicz 1997).

In pregnant PCOS women, when insulin levels are significantly increased, maternal androgen production via increased ovarian P450c17α activity may be further stimulated. The resulting maternal hyperandrogenism potentially overwhelms the ability of the placenta to convert androgens to estrogens, thereby exposing the fetus to androgen excess. Kavoussi *et al.* have demonstrated that the programming of adulthood insulin resistance is due to the androgenic effects of testosterone, rather than its conversion to estrogen in the placenta, in the ovine model of prenatal androgen excess (Kavoussi *et al.* 2006). By comparing insulin sensitivity indices at 18 months of age of prenatally testosterone (T)- and dihydrotesterone (DHT)-treated ewes, they found that insulin sensitivity was lower in the treatment groups when compared to controls (Kavoussi *et al.* 2006). This occurred with no difference in metabolic clearance rates of insulin. The elevated androgens available during fetal development and differentiation act as potent gene transcription factors and induce other transcription factors that can permanently alter mRNA concentrations and reprogram multiple organ systems (Abbott *et al.* 2002; Auger *et al.* 2001). These changes persist into postnatal life and lead to adulthood diseases.

In addition to the elevated levels of circulating androgen, an increase in the sensitivity of the androgen receptor (AR) may also occur in PCOS women. Outside of the reproductive system, the AR has been demonstrated to be present in cardiomyocytes, vascular smooth muscle, the endothelium, and kidney as well as others (Quinkler *et al.*

2005; Czubryt *et al.* 2006; Takeda *et al.* 1990). Activation of the AR has been shown to regulate gene expression, both positively and negatively, via different modes of action. Direct modulation of gene expression occurs upon androgen binding to the receptor in the cytoplasm and causing translocation to the nucleus. In the nucleus, the AR then binds to DNA via zinc fingers and interacts with androgen response elements of target genes and recruits other transcriptional machinery (Czubryt *et al.* 2006; Peterziel *et al.* 1999). This genomic signaling represents the method through which androgens program target cells, organs, and tissues to carry out complex functions (Simoncini and Genazzani 2003). Indirect signaling occurs through the interaction of the ligand-bound AR with various cytoplasmic signaling pathways, such as the MAPK, Akt, PKA, and PKC pathways, and increasing the free intracellular calcium levels (Kousteni *et al.* 2001; Heinlein and Chang 2002; Manin *et al.* 2002; Peterziel *et al.* 1999; Sharma *et al.* 2002). In addition, the non-genomic effects have also been reported to occur without acting through a receptor, as well as acting through other non-steroid receptors (Cato *et al.* 2002). The various signaling pathways that interact with the androgen receptor are summarized in Figure 1.

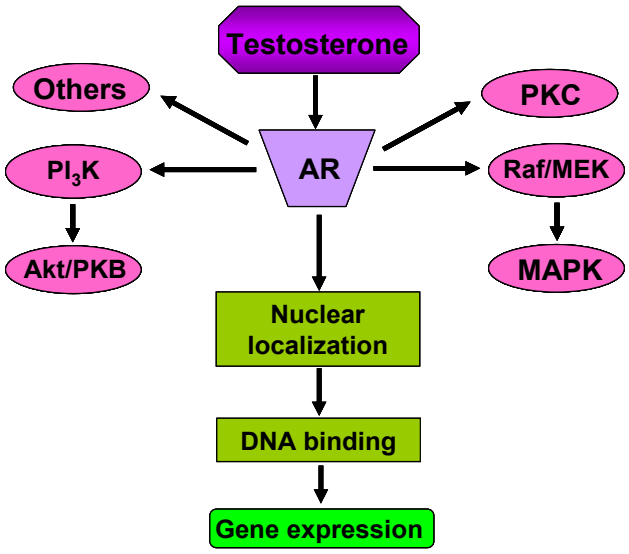


**Figure 1. Summary of androgen receptor signaling through various pathways.**

A major result of signals generated by androgens in these various systems is the activation of various kinases and the consequential modification of the phosphorylation state of important regulatory proteins and transcription factors (Watson and Gametchu 1999). This indirect interaction with various components of the signaling pathways provides a rapid, non-genomic method of effecting cell function and activity that allows the cell to adapt to dynamic changes, in addition to modulating long term processes such as gene expression, protein or DNA synthesis, and cell proliferation (Simoncini and Genazzani 2003; Castoria *et al.* 1999). In fact, the rapid actions are an integral part of the genomic actions and play an important role in physiological and pathophysiological processes (Cato *et al.* 2002). It also provides a possible link between excess androgens and alterations in metabolism, cardiovascular development and function, the central nervous system, and insulin resistance, especially in tissues that have been considered non-traditional targets of steroid hormones (Simoncini and Genazzani 2003; Czubryt *et al.* 2006). Previous studies have shown that androgens stimulate cardiac hypertrophy by a receptor-specific mechanism (Li *et al.* 2004; Marsh *et al.* 1998) and that they regulate the functional expression of an L-type calcium channel in ventricular myocytes that can alter cardiac performance (Golden *et al.* 2003). Reckelhoff *et al.* have also provided support for testosterone stimulation of the renin-angiotensin system and for causing the development of hypertension due to a hypertensive shift in the pressure-natriuresis relationship (Reckelhoff and Granger 1999; Reckelhoff 2001). One possible method may be by regulating the expression of epithelial sodium channels in the kidney (Quinkler *et al.* 2005).

There is evidence that the activity of the AR is determined by polymorphisms in the AR gene. Within exon 1 of the AR gene, there is a polymorphic region containing variable CAG repeats that encodes for a polyglutamine tract in the N-terminal domain (Xita *et al.* 2002; Mifsud *et al.* 2000; Carson-Jurica *et al.* 1990). Short polyglutamine tracts show high intrinsic AR activity, where as long tracts have a decreased capacity to activate androgen-responsive genes (Mifsud *et al.* 2000; Mhatre *et al.* 1993; Tut *et al.* 1997). Hsiao *et al.* have suggested that the molecular mechanism responsible for altered AR activity due to variable polyglutamine length is due to a nuclear G-protein that acts as a coactivator with the AR and that this G-protein binds differentially with different polyglutamine lengths (Hsiao and Chang 1999; Mifsud *et al.* 2000). Increased binding occurs with shorter repeats. In a subset of PCOS women who are not hyperandrogenemic but have AR hypersensitivity, Mifsud *et al.* showed the presence of short polyglutamine repeats in the AR (Mifsud *et al.* 2000). If other subsets of PCOS women are also found to have short polyglutamine repeats, it may explain why excessive androgens have a greater role in PCOS than expected from simple hyperandrogenism (Mifsud *et al.* 2000). While the cause of this polymorphism is not known, there has been shown to be racial and ethnic variations in the frequency of the CAG allele (Edwards *et al.* 1992). This variation may help to explain some of the heterogeneity seen in PCOS, but more research needs to be done to clarify the role that AR polymorphisms play in the pathogenesis of PCOS (Xita *et al.* 2002).

**Fetal Environment and Cardiovascular Development**

During fetal development, formation of the vascular network lays the foundation for adequately perfusing the growing fetus. Alterations in the regulation of this network

can affect the growth and development of various fetal tissues and organs, as well as later in life, and have serious implications to the health of an individual. A reduction in angiogenesis can lead to an altered vascular network that leads to increased peripheral resistance, hypertension, and potentially end-organ damage. The consequences of such fetal changes may not be noticeable until later in life.

The angiopoietins play a critical role in the assembly and maintenance of the cardiovascular system. Angiopoietin 1 and 2 (Ang1 and Ang2) act through their receptor, tunica interna endothelial cell kinase-2 (Tie2), to stimulate growth and remodeling of the cardiovascular system via antagonist pathways. Ang1 is a key mediator for the development of new blood vessels and for stabilizing existing vessels (Yamakawa *et al.* 2004; Hagen *et al.* 2005). Ang2, on the other hand, is an antagonist to Ang1 and works to destabilize existing vessels to aid in sprout formation and branching (Maisonpierre *et al.* 1997). In the absence of other angiogenic factors, specifically vascular endothelial growth factor, Ang2 causes endothelial cell apoptosis and vascular regression (Yamakawa *et al.* 2004). In fact, over-expression of Ang2 in transgenic mice is fatal during embryogenesis (Maisonpierre *et al.* 1997). During normal development, a balance between Ang1 and Ang2 allows for the regulated destabilization of existing vessels by Ang2 to allow branching and the stabilization of existing and new branches through the actions of Ang1. Up-regulation of Ang1, with down-regulation of Ang2, stabilizes the existing vasculature and reduces angiogenesis. The pathways and actions of Ang1 and Ang2 are summarized in Figure 2. Characterizing the changes in Ang1, Ang2, and Tie2 over time should help to support the hypothesis of developing

hypertension proposed in response to the changes seen in cardiac and renal weights associated with fetal exposure to excess testosterone.

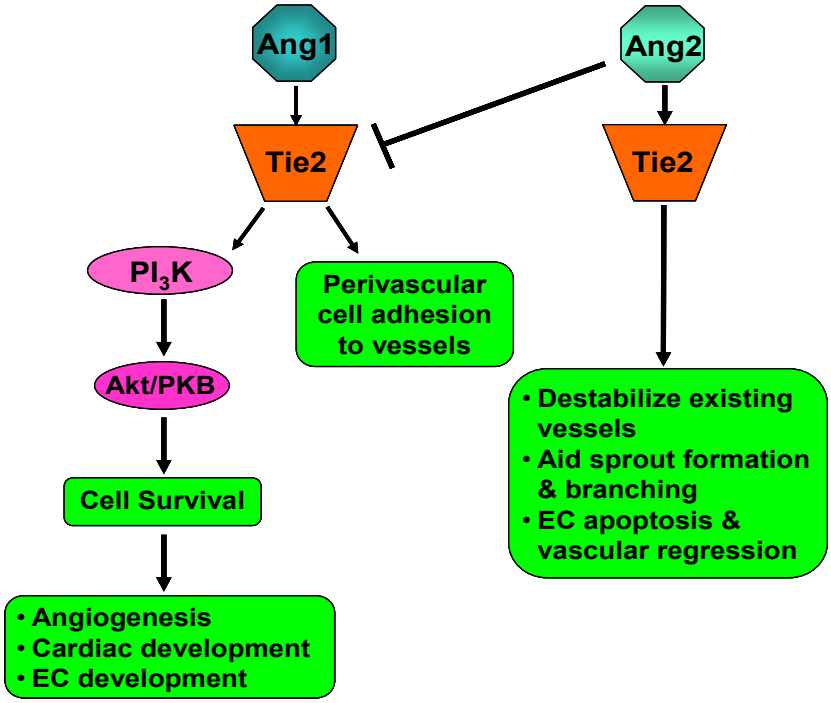


**Figure 2. Summary of angiopoietin 1 & 2 mechanism of action via Tie2.**

Another important angiogenic mediator is vascular endothelial growth factor (VEGF). VEGF is an endothelial-specific growth factor that induces proliferation, migration, sprouting and tube formation of endothelial cells (Kroll and Waltenberger 1998; Hiratsuka *et al.* 2005; Tammela *et al.* 2005), and is one of the most potent factors associated with fetal vascular growth in a number of species (Vonnahme *et al.* 2005). VEGF binds to two receptor-type tyrosine kinases, Flt-1 (VEGF R1) and KDR/Flk-1 (VEGF R2) to regulate both physiological and pathological angiogenesis (Zachary and Gliki 2001; Shibuya and Claesson-Welsh 2006). The actions of VEGF, through its interaction with these two receptors, are mediated by a variety of second messenger pathways in a tissue-dependent manner. VEGF R1 and R2 are differentially expressed in

vascular endothelial cells with VEGF R2 being the major positive signal transducer for all angiogenic states (Shibuya 2006; Kanno *et al.* 2000; Zachary and Gliki 2001; Shibuya and Claesson-Welsh 2006). It is most highly expressed during early embryogenesis and declines during later stages of vascular development (Shibuya and Claesson-Welsh 2006). R2 signaling is essential for the differentiation of endothelial precursor cells into vascular endothelial cells and their proliferation (Shibuya 2006). In fact, R2 knockout mice die by embryonic day 8.5 due to a lack of endothelial cells and hematopoietic cells (Kanno *et al.* 2000). R1 signaling, on the other hand, is relatively weak with mild biological activity that may act as a negative regulator of R2 (Shibuya 2006; Zachary and Gliki 2001). During embryogenesis, R1 most likely suppresses pro-angiogenic signals that are responsible for coordinated growth and organization of blood vessels as R1 null mice die around embryonic day 8.5 due to overgrowth of endothelial cells which do not assemble into functional blood vessels (Kanno *et al.* 2000; Fong *et al.* 1995). In adulthood, R1 stimulates inflammation, tumor growth, and metastasis (Shibuya 2006) and soluble R1 levels are elevated in preeclampsia patients (Shibuya and Claesson-Welsh 2006). Figure 3 summarizes the mechanism of action and pathways involved in VEGF signaling via R1 and R2.

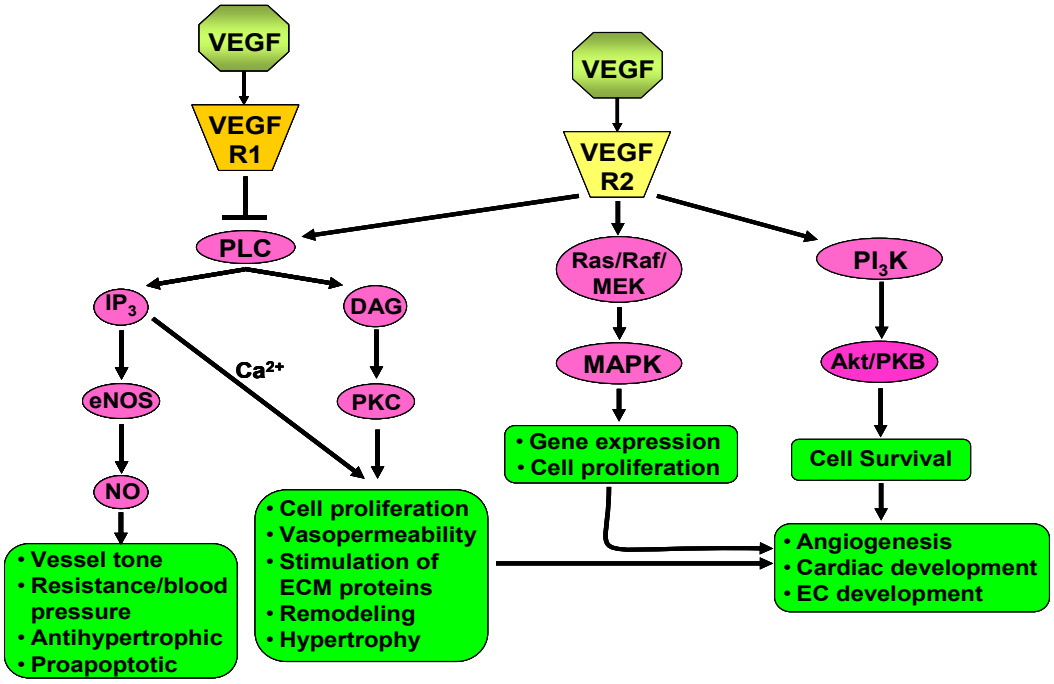


**Figure 3. Summary of vascular endothelial growth factor mechanism of action.**

In addition to its own direct effects, VEGF also interacts with Ang2 to promote vascular sprouting, as mentioned above, and works synergistically with Ang1 to stimulate angiogenesis (Koblizek *et al.* 1998). VEGF also stimulates an increase in endothelial nitric oxide synthase (eNOS) concentration and subsequent nitric oxide production by endothelial cells (Vonnahme *et al.* 2005). Changes in the concentration of VEGF during fetal development could have potentially long-term effects on the cardiovascular system. Down-regulation during fetal periods when the vascular network is being formed could lead to long-term alterations in gene expression and vascular development that could contribute to increased peripheral resistance and hypertension later in life. Also, reduced VEGF stimulation of eNOS could potentially lead to less responsive vessels and further increase peripheral resistance.

Nitric oxide (NO) is an important vasodilator and is synthesized from L-arginine by three isoforms of nitric oxide synthase (NOS): inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), and endothelial nitric oxide synthase (eNOS). While iNOS is activated in response to stress conditions, both eNOS and nNOS are constitutively expressed in a variety of tissues (Belge *et al.* 2005). Endothelial nitric oxide synthase is synthesized in and released by the endothelial cells to regulate vascular smooth muscle tone and blood flow. Deficiencies in eNOS have been implicated in the pathogenesis of hypertension, atherosclerosis, and preeclampsia (Celermajer and Sorensen 1992; Cockell and Poston 1997; Senthil *et al.* 2005; Celermajer *et al.* 1994). Endothelial dysfunction alters the regulation of NO production and affects cardiovascular function, as well as impairing the function of biologically active substances, such as insulin, by altering their transcapillary passage to target tissues. In addition to its metabolic effects (discussed later), insulin has non-metabolic actions targeted at cardiovascular hemodynamics, many of which center on its ability to stimulate NO synthesis and release. Insulin stimulates the recruitment of capillaries, causes peripheral vasodilation, and increases regional blood flow (Cersosimo and DeFronzo 2006; Baron 1994; Liang *et al.* 1982; Vincent *et al.* 2004). Insulin's hemodynamic effects work primarily by increasing the availability of nitric oxide from endothelial cells (Cersosimo and DeFronzo 2006; Zeng and Quon 1996; Vincent *et al.* 2003; Shaul 2002). Figure 4 summarizes the hemodynamic and metabolic actions of insulin (Cersosimo and DeFronzo 2006).

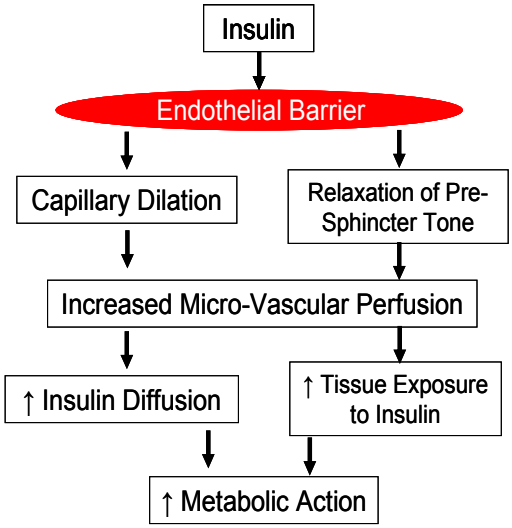


**Figure 4. Hemodynamic and metabolic actions of insulin (Cersosimo and DeFronzo 2006).**

Endothelial cell dysfunction and the subsequent resistance to insulin results in reduced nitric oxide production and is one factor that leads to impaired capillary expansion and blood flow to metabolically active tissues (Cersosimo and DeFronzo 2006). This may be part of the reason for the death of diabetic tissues. In the kidney, renal circulation is dependent on tonically released NO and inhibition of NOS results in a significant decrease in renal blood flow (Kihara *et al.* 2005). This has potential consequences on systemic blood pressure and may lead to the development of hypertension.

One of the most important regulators of cardiovascular development and hemodynamic control is the renin-angiotensin system (RAS). The RAS plays an important role during fetal development as well as throughout postnatal life. Angiotensin II is a potent vasoconstrictor and regulates a variety of biological processes including blood pressure, fluid homeostasis, sympathetic signaling, vascular smooth muscle tone, and cellular growth (Watanabe *et al.* 2005; Zheng *et al.* 2005; Huckle and Earp 1994;

Unger *et al.* 1996). The actions of angiotensin II are mediated primarily through two receptor subtypes. The type I (AT1) receptor is believed to be responsible for most of the known angiotensin II effects, whereas the type 2 (AT2) receptor is less well defined but is thought to act as an antagonist to AT1 effects on blood pressure and cell proliferation (Unger *et al.* 1996; Huckle and Earp 1994; Zheng *et al.* 2005). In addition to the antagonistic actions during postnatal life, AT2 is thought to be important in cardiac development during fetal life (Watanabe *et al.* 2005). In the myocardium and vascular smooth muscle, AT1 activation has been shown to stimulate hypertrophy and increased contractility (Timmermans *et al.* 1992). AT1 activation in the kidney leads to constriction of the afferent and efferent arterioles (Timmermans *et al.* 1992). The combination of these actions on the cardiovascular system and kidney potentially contribute to the development of systemic hypertension. In spontaneously hypertensive rats, inhibition of AT1 with losartan lowered blood pressure and reversed the vascular hypertrophy seen in untreated spontaneously hypertensive rats (Timmermans *et al.* 1992). Figure 5 summarizes the actions of angiotensin II through its interactions with AT1 and AT2.

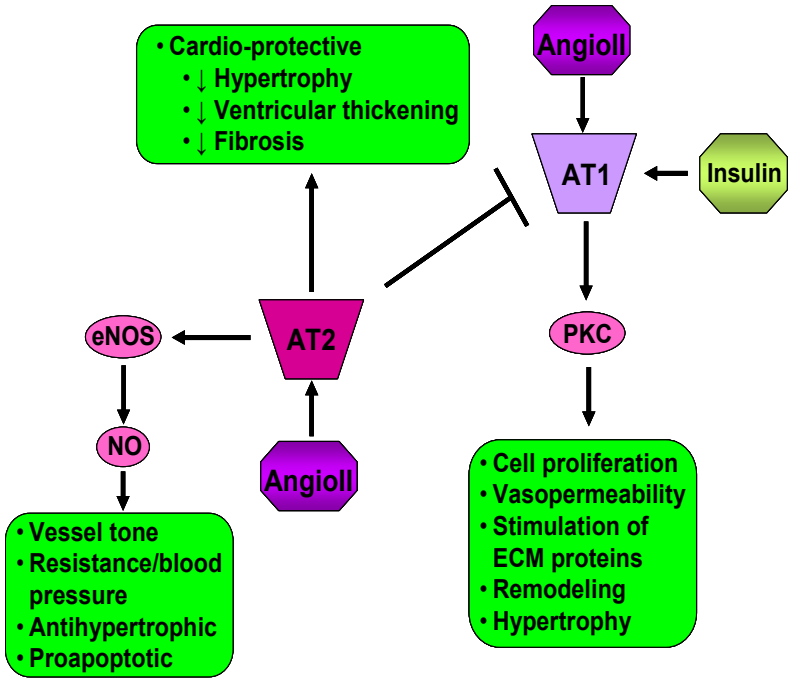


**Figure 5. Summary of angiotensin II mechanism of action.**

Like VEGF, angiotensin II can also regulate NO production. Angiotensin II regulation of NO production has been reported to occur via the AT2 receptor in cultured cardiomyocytes and aortic endothelial cells, while AT1 receptors appear more important in stimulating NO production from cultured pulmonary and coronary arteries (Leite-Moreira *et al.* 2006; Kihara *et al.* 2005). In the kidney, this most likely occurs as a renal protective mechanism to protect renal blood flow to the vasoconstrictive action of angiotensin II and angiotensin II-induced hypertension.

The formation, maintenance, and regulation of the cardiovascular system involve the complex interaction of numerous mediators. Alterations in their presence or concentration, especially during critical periods of system development can potentially have broad consequences on cardiovascular hemodynamics and overall health. Figure 6

briefly summarizes the actions and interactions of a select number of cardiovascular mediators.

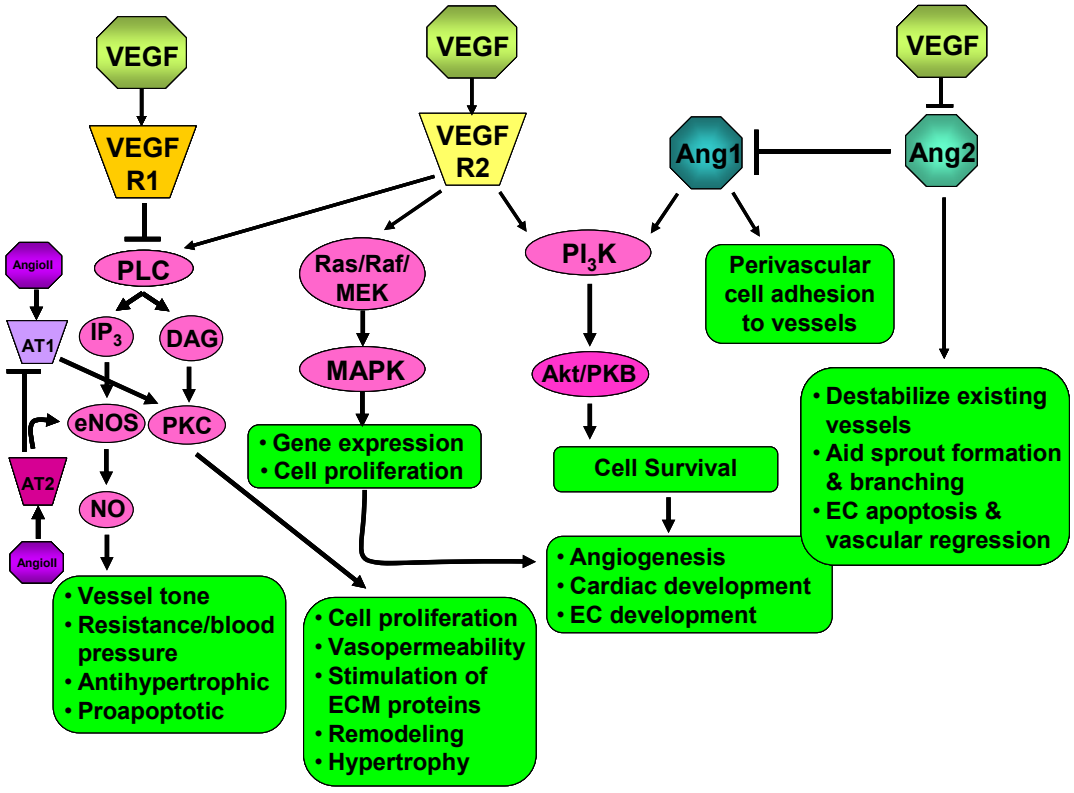


**Figure 6. Summary of cardiovascular actions and interactions of selected mediators.**

## Fetal Environment and Metabolic Regulation

The hemodynamic effects of insulin discussed previously are closely associated with its metabolic effects. Insulin is a powerful anabolic hormone that plays an important role as a growth factor, in glucose metabolism, and in hormonal and metabolic regulation. Insulin initiates its actions on target cells by first binding to a membrane receptor. The insulin receptor is comprised of four subunits, 2 α subunits on the extracellular surface and 2 transmembrane β subunits. Insulin binding with the α subunits causes auto-phosphorylation of the β subunits, activating intrinsic tyrosine kinases that subsequently phosphorylate other intracellular messengers (Figure 7).

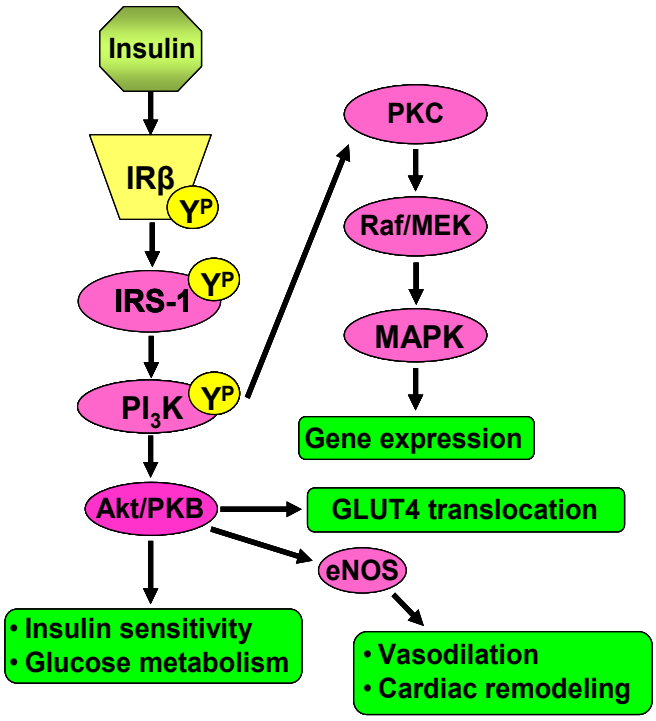


**Figure 7. Normal insulin signaling.**

Insulin signaling promotes glycogen synthesis in liver and muscle, triglyceride synthesis and deposition in adipose tissue, increases protein synthesis, and inhibits proteolysis (Cersosimo and DeFronzo 2006). It also promotes the oxidation of glucose during glycolysis, promotes the translocation of GLUT4 to the cell membrane, acts as a growth factor, regulates gene expression, and stimulates the production of such steroid hormones as androgens. As insulin works to increase blood flow to target tissues, it is able to increase the availability of insulin to those tissues. However, under certain conditions, the actions of insulin are impaired as cells become insulin resistant. When tissues become more resistant to the metabolic effects of insulin, the normal response for the pancreatic β cells is to increase insulin production and secretion (Cersosimo and DeFronzo 2006). This elevated level of unusable insulin may then have unintended

consequences on the overall health of an individual. The exact cellular mechanisms involved in the various actions of insulin are still developing and are not fully understood.

Insulin's interaction with other hormones also plays a critical role in its signaling. In addition to its cardiovascular effects, a role appears to exist for angiotensin II in glucose metabolism through its interaction with insulin receptor signaling. As discussed earlier, normal insulin signaling involves phosphorylation of various tyrosine residues within the signaling cascade (Figure 7). Folli *et al.* have demonstrated in isolated rodent smooth muscle cells that angiotensin II stimulation leads to a 60% decrease in insulin signaling by inducing serine phosphorylation and subsequently decreasing the association of the insulin receptor β subunit (Folli *et al.* 1997) (Figure 8).

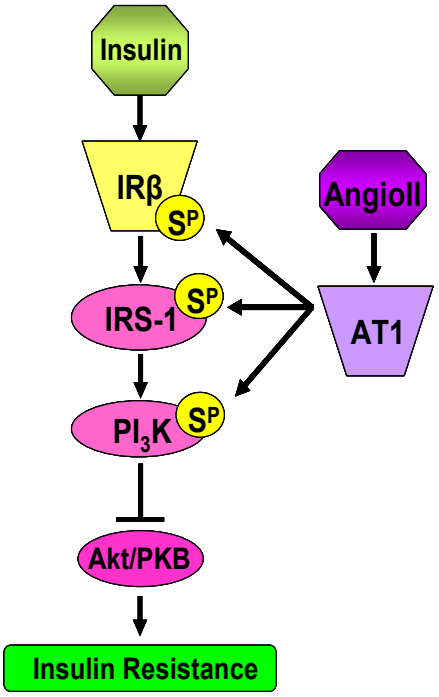


**Figure 8. Angiotensin II action on insulin signaling.**

At this time, it is not known whether there is a genetic basis for the increase in serine phosphorylation of the insulin receptor β subunit, especially in women with polycystic ovary syndrome (PCOS); however, nearly 50% of these women have insulin receptors with higher levels of serine phosphorylation (Ibanez *et al.* 1998; Xita *et al.* 2002). This may potentially explain the higher levels of insulin resistance and elevated androgen levels in this population of women. There is evidence that the factor causing the increased serine phosphorylation is extrinsic to the insulin receptor (Xita *et al.* 2002) and angiotensin II is one extrinsic factor whose interaction has been shown to alter normal insulin signaling. Improved glucose tolerance following administration of angiotensin II antagonists has been demonstrated in adults with peripheral insulin resistance (Katovich *et al.* 2001; Ogihara *et al.* 2002). In rodents with obesity, hypertension, and insulin resistance, angiotensin II antagonists have improved insulin sensitivity (Ogihara *et al.* 2002; Richey *et al.* 1999; Juan *et al.* 2005).

**Possible Mechanisms for Fetal Programming in Polycystic Ovary Syndrome**

While the mechanisms underlying androgen excess leading to fetal programming of the PCOS phenotype are not well understood, Xita *et al.* present three possible biological mechanisms (Xita and Tsatsoulis 2006). The first, and most likely, is altered target tissue differentiation due to exposure during critical periods of development. Alterations to metabolic regulation and control in fetal life may become permanent, increasing the risk of developing the metabolic syndrome as an adult. The second possible mechanism is via epigenetic changes in gene expression. However, to date there is no evidence that this occurs as a result of prenatal androgen excess. The third possible mechanism proposed by Xita *et al*. is that the fetus undergoes an adaptive homeostatic

process that confers a survival advantage (Xita and Tsatsoulis 2006). This has been shown in response to fetal under-nutrition (Lindstedt *et al.* 1991), but remains speculative to whether it also applies to PCOS.

There is strong and growing evidence to support fetal programming of female fetuses and the development of the PCOS phenotype in response to elevated androgens during fetal life. Genetic and environmental factors may act independently in the development of PCOS, but more likely it is the interaction between the two that is responsible for the heterogeneity of expression seen in women with polycystic ovary syndrome.

# CHAPTER III

## Fetal Androgen Exposure Alters Angiopoietin 1, Angiotensin II Receptor Subtypes 1 and 2, eNOS, VEGF, and VEGF Receptor Expression

### Introduction

During fetal development, tissues and organs undergo critical periods of development that coincide with periods of rapid cell division (Barker 2001). Disturbances during these critical periods can influence homeostatic mechanisms, the effects of which may be subtle during development, but have long-lasting negative impacts on adult health (Hoet and Hanson 2001). In growth restricted fetuses, short-term adaptations are beneficial for fetal survival but are often detrimental to health later in life. These adaptations can permanently alter structure, function, and metabolism. Adulthood diseases linked to fetal growth restriction and fetal programming include cardiovascular diseases, hypertension, and type II diabetes.

Polycystic ovary syndrome (PCOS) is an endocrine disorder affecting 5 – 10% of reproductive age women and displays a wide phenotypic heterogeneity (Sir-Petermann *et al.* 2002b; Sir-Petermann *et al.* 2002a; Xita *et al.* 2002). Women with PCOS often display signs of the metabolic syndrome, such as hyperinsulinemia, type II diabetes, peripheral insulin resistance, cardiovascular disease, hypertension, and dyslipidemia (Sir-Petermann *et al.* 2002a; Sir-Petermann *et al.* 2005; Orio *et al.* 2006). Up to half of all PCOS women meet the criteria for the metabolic syndrome, with insulin resistance implicated as the pathological link between the two syndromes (Essah *et al.* 2007).

While the development of PCOS symptoms typically does not occur until the onset of puberty, the underlying causes are most likely present since birth, or earlier.

Manikkam *et al.* have shown that an ovine model of PCOS leads to fetal growth restriction (Manikkam *et al.* 2004) and a follow-on evaluation of female offspring at 21 months of age indicated increased cardiac and kidney weights in the prenatal testosterone treated ewes, suggesting the development of systemic hypertension (Padmanabhan *et al.* 2004). This model has clinical relevance as women with PCOS also develop signs of the metabolic syndrome and have a higher incidence of fetal growth restricted offspring. Until recently, this model has focused primarily on the hypothalamic-pituitary-gonadal axis during prepubertal development and adult reproductive life (Recabarren *et al.* 2005).

Recent studies have begun to evaluate the metabolic effects of glucose metabolism and insulin resistance (Eisner *et al.* 2000; Recabarren *et al.* 2005; Kavoussi *et al.* 2006) using the ovine PCOS model, but little attention has been given to the cellular mechanisms involved in the development of the metabolic syndrome commonly found in PCOS women. Overall, similarities between the sheep model and PCOS women suggest common mediators between androgen-induced fetal growth restriction and fetal programming that lead to the cardiovascular and metabolic disturbances later in life.

During fetal development, formation of the vascular network lays the foundation for adequately perfusing the growing fetus and involves the complex interaction of numerous mediators. Reduced angiogenesis can alter the vascular network and lead to increased peripheral resistance, hypertension, and potentially end-organ damage. The consequences of such fetal changes may not be noticeable until later in life.

The endothelial-specific growth factor vascular endothelial growth factor (VEGF) induces proliferation, migration, sprouting and tube formation of endothelial cells (Kroll and Waltenberger 1998; Hiratsuka *et al.* 2005; Tammela *et al.* 2005), and is one of the most potent factors associated with fetal vascular growth in a number of species (Vonnahme *et al.* 2005). The actions of VEGF, through interaction with the Flt-1 (VEGF R1) and KDR/Flk-1 (VEGF R2) receptors, are mediated by a variety of second messenger pathways in a tissue-dependent manner. Angiopoietin 1 and 2 (Ang1 and Ang2), acting through the tunica interna endothelial cell kinase-2 (Tie2) receptor, play a critical role in cardiovascular development via antagonist pathways. During normal development, a balance between Ang1 and Ang2 regulates destabilization of existing vessels by Ang2 to allow branching of existing vessels and stabilization of new branches through the actions of Ang1.

Endothelial nitric oxide synthase (eNOS) is synthesized and released by endothelial cells to regulate a number of cardiovascular functions. Endothelial dysfunction and deficiencies in eNOS have been implicated in the pathogenesis of hypertension, atherosclerosis, and preeclampsia (Celermajer and Sorensen 1992; Cockell and Poston 1997; Senthil *et al.* 2005; Celermajer *et al.* 1994). The renin-angiotensin system is an important cardiovascular regulator and plays an important role throughout fetal development and postnatal life. Angiotensin II, acting through antagonistic receptor subtypes AT1 and AT2, is a potent vasoconstrictor and regulates a variety of biological processes including blood pressure, fluid homeostasis, sympathetic signaling, vascular smooth muscle tone, and cellular growth (Watanabe *et al.* 2005; Zheng *et al.* 2005; Huckle and Earp 1994; Unger *et al.* 1996).

The combination of these mediators' actions on the cardiovascular system and kidney potentially contribute to the development of systemic hypertension. We hypothesized that prenatal exposure to excess testosterone from days 30 to 90 of gestation will up-regulate the mRNA and protein concentrations of angiogenic growth factors and mediators of insulin signaling and glucose metabolism in treated fetal cardiac and kidney tissues and then decrease postnatally in an age-dependent manner.

## Methods & Materials

### *Animal Care*

Adult Suffolk ewes with proven fertility were purchased and housed at a USDA-inspected and University of Michigan Department of Laboratory Animal Medicine-approved farm for breeding. To increase energy balance in an attempt to increase ovulation rates, ewes were group-fed daily with 0.5 kg shelled corn and 1.0 – 1.5 kg alfalfa hay/ewe from 2 to 3 weeks before breeding, continuing up to breeding. After breeding, all ewes were housed under natural photoperiod in a pasture and group-fed with a daily maintenance diet of 1.25 kg alfalfa/brome hay mix per ewe. Pregnant ewes were assigned to either a control (n = 16) or a prenatal testosterone treatment (n = 32) group. The treatment group ewes received twice weekly im injections of 100 mg testosterone (T)-propionate in 2.4 ml cottonseed oil from days 30 – 90 of gestation (term = 147). The control ewes received im injections of vehicle only. Lambs were weaned at 8 weeks of age and all female lambs were transferred to the Sheep Research Facility (Ann Arbor, MI) where they were provided *ad libitum* access to commercial feed pellets and monitored for postnatal gain. At approximately 40 kg, all lambs were switched to a pellet

feed with 15% crude protein to avoid fat deposition. Trace mineralized salt with selenium and vitamins A, D, and E (Armada Grain Co., Armada, MI) were freely accessible throughout the study (Manikkam *et al.* 2004). A sub-set of control and growth restricted prenatally T-treated treated females were maintained until either 9 months or 21 months of age (Padmanabhan *et al.* 2004).

***Tissue Collection***

The tissues analyzed in the current study were collected from a subset of offspring from a study originally conducted by Manikkam *et al.* to determine whether prenatal testosterone treatment produced growth-retarded offspring (Manikkam *et al.* 2004). Heart and kidney tissues were collected from female offspring at 65 dGA (con = 8; treat = 9) and 90 dGA (con = 8; treat = 8) and frozen in liquid nitrogen for later analysis. In addition, two groups of offspring were maintained until 9 months (con = 6; treat = 5) or 21 months (con = 8; treat = 8) of age for tissue harvest and analysis. Adult heart tissue was dissected into left ventricle + septum and right ventricle then weighed. The left ventricle and septum were then separated, and left ventricle, right ventricle, and septum were frozen in liquid nitrogen and stored at -80°C. Adult kidney sections were also frozen in liquid nitrogen and stored in the same manner as the cardiac samples. For all samples, birth/fetal weight, body weight at time of tissue collection, and tissue weights were recorded.

***Tissue Dry Weights***

Tissue dry weights were determined by weighing out 100 mg of frozen tissue into labeled weigh boats of known weight. Each sample was weighed out in duplicate and placed into a 100°C oven to dry. At the end of seven days, samples were removed from

the oven one at a time, immediately weighed, and returned to the oven. Samples remained in the oven overnight and were reweighed the next day. Each sample was weighed a total of three times and the average dry weight calculated. The percent dry weight was calculated by dividing the tissue dry weight by the starting weight and multiplying by 100. Dry weights were determined for all 9 and 21 month left ventricle, right ventricle, and kidney samples. Due to the limited amount of fetal tissue available, fetal dry weights were not determined.

***Total Cellular RNA Extraction and Quantification***

Total cellular RNA (tcRNA) was isolated from all fetal and adult cardiac tissues using the Qiagen RNeasy® Fibrous Tissue kit. The Qiagen RNeasy® kit was used to isolate tcRNA from all fetal and adult renal tissues. For both kits, the manufacture's instructions were followed with slight modification. Briefly, 30 mg of powdered tissue was added to a chilled 1.7 ml centrifuge tube, 300 – 600 µl of Buffer RLT from the kit added, and the sample vortexed for 60 seconds, or until completely homogenized. The sample was then placed in a QiaShredder (Qiagen) and centrifuged for 2 minutes at maximum speed. The sample was then treated according to manufacturer's instructions until the final tcRNA recovery step. For cardiac tissues, samples were DNase treated per kit instructions. tcRNA was eluted off the column using 100 µl of RNase-free water, collected via centrifugation, followed by elution with another 100 µl of RNase-free water and collection via centrifugation. The recovered eluate was then precipitated overnight using 100% ethanol at -20°C. tcRNA was quantified on the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.) by measuring the $A_{260}/A_{280}$ ratio, the volume needed for 5 µg tcRNA calculated, and multiple aliquots made for later reverse

transcription reactions in order to avoid repeated freeze-thaw of tcRNA samples. All samples were stored at -80°C.

***Reverse Transcription cDNA Synthesis***

For the reverse transcription reaction, 5 μg of tcRNA was used in the SuperScript™ III First-Strand Synthesis System for RT-PCR kit (Invitrogen). The manufacturer's protocol for first strand cDNA synthesis of transcripts with high GC content, using oligo$(dT)_{20}$ primers, was used as it resulted in better yields than the normal kit protocol. The main difference between the protocols was the use of a larger reaction volume (50 μl vs. 25 μl). cDNA quantification was performed using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.) by measuring the $A_{260}/A_{280}$ ratio. The cDNA was diluted to 50 ng/ul using DepC-treated water and aliquots of diluted cDNA made and stored at -20°C for later real-time PCR analysis. All aliquots of diluted cDNA were analyzed within 7 days of the initial dilution.

***Primer Design***

Gene-specific forward and reverse primers were designed for the following genes to be analyzed via real-time PCR: angiopoietin 1 (Ang1), angiopoietin 2 (Ang2), tunica interna endothelial cell kinase-2 (Tie2), angiotensin II receptor type 1 (AT1), angiotensin II receptor type 2 (AT2), endothelial nitric oxide synthase (eNOS), ribosomal protein s15 (s15), RNA polymerase II (Pol II), vascular endothelial growth factor (VEGF), VEGF receptor 1 (R1), and VEGF receptor 2 (R2). All primers were designed from published gene sequences and were designed to span at least one intron to distinguish genomic contamination. Primer sequences are listed in Table 1 and sequences for each gene are listed in Attachment 1.

The optimal annealing temperature for each primer set was determined by performing traditional PCR using the *Taq* DNA Polymerase kit (Invitrogen) and amplifying over a temperature gradient. The optimal temperature was determined to be the highest temperature that yielded a significant quantity of a single amplicon as determined by gel electrophoreses and ethidium bromide quantification. After determination of the optimal temperature for each gene, PCR was performed using cDNA generated using tcRNA from the samples. The PCR product was purified and ligated into the pScript-Amp SK(+) cloning vector (Stratagene), transformed into XL10-Gold® Kan ultra-competent cells (Stratagene), and amplified. The plasmids were then purified by alkaline lysis and ultracentrifugation on a cesium chloride gradient and sequenced by Macromolecular Resources at Colorado State University.

**Table 1. Summary of Ovine Primers used in Real-Time PCR Analysis of Cardiovascular Mediators.**

| Gene | Forward Primer | Reverse Primer | Product Length (bp) | Temp (°C) | Accession Number |
|---|---|---|---|---|---|
| **Ang1** | ttgccataaccagtcagag | aaccaccagcctcctgtta | 259 | 60 | AY881028 |
| **Ang2** | gaccgctgtgatgatagaa | tttgattgacccgaagttga | 269 | 60 | AY881029 |
| **AT1** | gctgacttatgctttttact | ttccttggtttgttcttctg | 488 | 55 | NM_001009744 |
| **AT2** | gtttccaggatttacatctt | ggtcacgagttatcctattc | 616 | 55 | AF254444 |
| **eNOS** | tgcatgacattgagagcaaagggc | atgtcctcgtgatagcgttgctga | 391 | 60 | DQ015701 |
| **s15** | atcattctgcccgagatggtg | tgctttacgggcttgtaggtg | 134 | 60 | NM_001018 |
| **Pol II** | agtccaacatgctgacggacatga | agccaagtgccggtaattgacgta | 332 | 60 | CD288457 |
| **Tie-2** | gaggacaggcaataaggatac | ggagacagaacacgaaggact | 489 | 61 | AY881030 |
| **VEGF** | ttgccttgctgctctacctt | gggcacacactccagacttt | 251 | 60 | AF071015 |
| **VEGFR1** | tctatagcaccaagagcgacgtgt | agtacaacctgacgaccgtgtttc | 195 | 60 | AF063657 |
| **VEGF R2** | ttccactgggaataccttcttcg | tccaccaaggattccatgccacta | 251 | 60 | XM_050674 |

### *Real-Time PCR Analysis*

Standard curves were generated for real-time PCR for all genes by using restriction enzymes to cut out the PCR product that was cloned as described above. Gene-specific primers were used to generate a specific PCR product using the *Taq* DNA Polymerase kit (Invitrogen). The gene-specific PCR product was then purified using the PCR Purification Kit (Stratagene) and quantified by measuring the $A_{260}/A_{280}$ ratio on the

NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). A portion of the purified gene-specific PCR product was diluted to 10 ng/µl in DepC-treated water and a serial dilution performed to make the standard curve (100 pg/µl to $10^{-7}$ pg/µl) used in real-time PCR. All standard curves were optimized by adjusting the $MgCl_2$ concentration to optimize the PCR efficiency.

Reverse transcription reactions were performed on all control and treatment samples as described above and used for the real-time PCR assay within 7 days. The starting quantity of cDNA for real-time PCR was optimized for each gene and for each tissue type so that the real-time product fell at approximately the center of the standard curve and all samples were run in triplicate. Real-time PCR was performed using iQ™ SYBR® Green Supermix (Bio-Rad) on the LightCycler® 480 Real-Time PCR Detection System (Roche). Using the 384-well thermal block, multiple tissues were run simultaneously for the same gene, thereby minimizing between-run differences. A housekeeping gene that showed no statistical difference between control and treatment groups was selected for each tissue type (i.e. cardiac or renal) to normalize against. All cardiac samples were normalized to the housekeeping gene RNA polymerase II, while all renal samples were normalized to the housekeeping gene ribosomal protein s15. Melt curves were analyzed to ensure lack of contamination and examples of the standards and samples electrophoresed on 2% agarose gels to check for the correct product size. Examples of both are shown in Attachment 2.

***Protein Extraction***

Frozen adult left ventricle, adult right ventricle, adult kidney, fetal total heart, and fetal kidney were powdered under liquid nitrogen and stored at -80°C. Due to the small

amount of fetal tissue available, protein extraction was only performed on adult cardiac and renal samples as previously described (Regnault *et al.* 2002). Briefly, approximately 100 mg of powdered tissue were added to 500 µl of lysis buffer (0.48 M Tris pH 7.4, 10 mM EGTA pH 8.6, 10 mM EDTA pH 8.0, 0.1 mM PMSF, 0.1 mM protease/phosphatase cocktail (Sigma P8340)) and sonicated. Total cellular protein concentration was determined by Bradford assay (BioRad Protein Assay) and the absorbance at 595 nm was determined in a spectrophotometer (Beckman DU 530). All samples were run in duplicate and the average absorbance was used to calculate the protein concentration.

***Western Blot Analysis***

Western immunoblot analysis was performed as previously described for left ventricle, right ventricle, and kidney samples for both the 9 and 21 month age groups (Regnault *et al.* 2002). For each sample, 50 µg of total protein was electrophoresed through NuPAGE 4 – 12% Bis-Tris gels (Invitrogen) for 1 hour at 200 volts (4°C) in 1X MOPS running buffer (100 mM MOPS, 100 mM Tris-HCl, 3.5 mM SDS, and 0.8 mM EDTA). The proteins were transferred to 0.45 µm nitrocellulose membrane (Osmonics, Inc.) in a Trans-Blot Electrophoretic Transfer Cell System (BioRad) at room temperature for 5 hours at 60 volts in modified NuPAGE transfer buffer (5% 20X NuPAGE Transfer Buffer, 0.1% NuPAGE antioxidant, 20% methanol, and 0.1% SDS). After transfer, membranes were blocked with 5% non-fat dry milk in 1X TBST (0.1% Tween20, 10 mM Tris, pH 8.0, and 150 mM NaCl) overnight at 4°C.

Membranes were incubated with antibodies (Santa Cruz Biotechnology) against the following cardiovascular proteins: angiopoietin 1 (sc-6320, 1:1000 & 1:20k), angiopoietin 2 (sc-7015, 1:900 & 1:20k), angiotensin II receptor type 1 (sc-1173 HRP-

conjugated, 1:2500), angiotensin II receptor type 2 (sc-9040 HRP-conjugated, 1:2000), endothelial nitric oxide synthase (sc-654 HRP-conjugated, 1:1000), insulin receptor β (sc-711 HRP-conjugated, 1:900), tie-2 (sc-324, 1:200 & 1:20k), and vascular endothelial growth factor (sc-152 HRP-conjugated, 1:200). Values in parenthesis represent product number, primary antibody dilution, and secondary antibody dilution, respectively. Vascular endothelial growth factor receptors 1 and 2 were not evaluated due to the lack of adequate antibodies at the time the current study was conducted. For all proteins, antibody concentrations, chemiluminescence substrate type, and exposure time were optimized. Protein bands were detected by chemiluminescence using SuperSignal® West Pico or Femto (Pierce, 34080 or 34095) and quantified by densitometry using ImageQuant® 5.0 software. Representative immunoblots are shown in Attachment 3.

***Statistical Analysis***

Primary outcome measures were wet vs. dry weights, mRNA concentration, and protein concentration. Statistical differences between prenatal treatments, for a given age, were determined by Students t-test with a p-value $\leq 0.05$ accepted as statistically significant. Unless otherwise noted, values were reported as mean ± sem.

## Results

### Tissue Weights

Preliminary studies by Manikkam *et al.* have demonstrated that exposure to prenatal testosterone treatment produced growth-restricted offspring (Manikkam *et al.* 2004). They reported that when all offspring were considered, prenatally testosterone (T)-treated offspring weighed less than controls when adjusted for the number of

offspring per ewe (Manikkam *et al.* 2004). The difference was still significant when the sexes were analyzed separately and adjusted for litter size. A follow-up study using the female control and growth-restricted prenatally T-treated females from the above study evaluated the effect of prenatal T-treatment on cardiac and organ development (Padmanabhan *et al.* 2004). At 21 months of age, there were no differences in weights of liver, lung, thyroid, and spleen of control and prenatally T-treated animals. As shown in Table 2, left ventricle + septum and kidney weights at 21 months of age were 13% and 17%, respectfully, greater in prenatal T-treated offspring, whereas right ventricle weights were not different (Padmanabhan *et al.* 2004).

**Table 2. Previously Reported Differences in Tissue Weight in 21 Month Offspring.**

| | Heart | Left Ventricle + Septum | Kidneys |
|---|---|---|---|
| **Controls** | 0.299 ± 0.01 | 0.167 ± 0.005 | 0.166 ± 0.007 |
| **Treatment** | 0.335 ± 0.012* | 0.190 ± 0.009* | 0.195 ± 0.009* |

Values are mean kg ± sem. * = p value < 0.05

When the previously reported differences (Padmanabhan *et al.* 2004) in tissue weights were normalized to body weight, the significance initially seen at 21 months of age in the heart, left ventricle + septum, and kidney was lost (Table 3), suggesting that they may be larger due to the treatment animals simply being larger at the time of tissue collection.

**Table 3. Normalized Tissue Weights in 21 Month Offspring.**

| | Heart | Left Ventricle + Septum | Right Ventricle | Kidneys |
|---|---|---|---|---|
| **Controls** | 0.0041 ± 0.0001 | 0.0024 ± 0.0001 | 0.0006 ± 0.0001 | 0.0023 ± 0.0001 |
| **Treatment** | 0.0043 ± 0.0002 | 0.0025 ± 0.0001 | 0.0007 ± 0.0001 | 0.0025 ± 0.0002 |

Values are mean ± sem.

In addition, while no differences between treatment groups were detected for the left ventricle + septum and kidney weights at 9 months of age, significance was seen in normalized heart and kidney weights (Table 4). However, no differences were found in

dry weights of left ventricle, right ventricle, or kidney at either 9 or 21 months of age (Table 5).

**Table 4. Normalized Tissue Weights in 9 Month Offspring.**

| | Heart | Left Ventricle + Septum | Right Ventricle | Kidneys |
|---|---|---|---|---|
| **Controls** | 0.0042 ± 0.0002 | 0.0025 ± 0.0002 | 0.0009 ± 0.0001 | 0.0024 ± 0.0001 |
| **Treatment** | 0.0034 ± 0.0002* | 0.0023 ± 0.0002 | 0.0007 ± 0.00004 | 0.0028 ± 0.0001* |

Values are mean ± sem. * = $p < 0.05$.

**Table 5. Tissue Dry Weights.**

| | | Control | Treatment | p value |
|---|---|---|---|---|
| **9 Month** | **Left Ventricle** | 17.34 ± 0.12 | 17.25 ± 0.11 | 0.577 |
| | **Right Ventricle** | 15.95 ± 1.61 | 14.02 ± 0.79 | 0.342 |
| | **Kidney** | 18.14 ± 0.47 | 19.47 ± 2.01 | 0.499 |
| **21 Month** | **Left Ventricle** | 23.35 ± 1.43 | 21.69 ± 0.28 | 0.274 |
| | **Right Ventricle** | 24.52 ± 1.33 | 22.80 ± 0.94 | 0.309 |
| | **Kidney** | 20.25 ± 1.08 | 20.81 ± 1.06 | 0.717 |

Values are percent dry weight calculated as (mg dry tissue ÷ mg wet tissue)*100.
Values reported as mean ± sem.

No differences in fetal weight were found between treatment groups at either fetal Day 65 or fetal Day 90. At Day 65, no differences were seen between treatment groups for heart weight normalized to fetal weight, but there tended to be a reduction in the normalized kidney weight of the T-treated fetuses ($p = 0.059$) (Table 6). By Day 90, however, no differences existed between treatment groups. Due to the small amount of fetal tissue available, dry weights were not measured.

**Table 6. Normalized Tissue Weights at Fetal Day 65.**

| | Heart | Kidneys |
|---|---|---|
| **Controls** | 0.0087 ± 0.0004 | 0.0117 ± 0.0003 |
| **Treatment** | 0.0083 ± 0.0006 | 0.0105 ± 0.0005* |

Values are mean ± sem. *: $p = 0.059$.

### Angiogenic and Vasculogenic Mediators

The angiogenic and vasculogenic mediators studied included: Ang1, Ang2, their receptor Tie2, VEGF, VEGF R1, and VEGF R2. Overall, there were no significant

differences in mRNA concentrations between the control and treatment groups at either of the fetal time points in whole heart homogenates (Table 7).

**Table 7. Summary of Angiogenic & Vasculogenic Mediators in Fetal Cardiac Samples: Real-Time PCR Results.**

| | | Control | Treatment | p value |
|---|---|---|---|---|
| Day 65 | Ang1 | 0.0015 ± 0.0003 | 0.0019 ± 0.0003 | 0.320 |
| | Ang2 | 0.0671 ± 0.0049 | 0.1017 ± 0.0191 | 0.144 |
| | Tie2 | 0.067 ± 0.0062 | 0.0948 ± 0.0155 | 0.118 |
| | VEGF | 3.0 E-06 ± 4.3 E-07 | 2.8 E-06 ± 2.4 E-07 | 0.710 |
| | VEGFR1 | 0.00126 ± 0.00012 | 0.00138 ± 0.00017 | 0.587 |
| | VEGFR2 | 0.0555 ± 0.0043 | 0.0582 ± 0.0082 | 0.763 |
| Day 90 | Ang1 | 0.0396 ± 0.0116 | 0.0579 ± 0.007 | 0.189 |
| | Ang2 | 0.0035 ± 0.0005 | 0.0034 ± 0.0006 | 0.837 |
| | Tie2 | 0.6398 ± 0.0995 | 0.6455 ± 0.0822 | 0.965 |
| | VEGF | 0.7695 ± 0.143 | 0.7235 ± 0.163 | 0.839 |
| | VEGFR1 | 0.031 ± 0.0030 | 0.029 ± 0.0035 | 0.680 |
| | VEGFR2 | 67.089 ± 5.724 | 64.008 ± 5.085 | 0.696 |

Values are starting quantity of gene (in pg) normalized to starting quantity of RNA Polymerase II (in pg). Values reported as mean ± sem.

Changes were detected as the offspring aged, with changes seen in 9 month left ventricles where VEGF R2 mRNA concentration showed a tendency ($p = 0.059$) to be elevated in the treatment group (Table 8). At 21 months of age, changes in angiopoietin 1 (Ang1) mRNA concentration in the treatment group were detected with a significant reduction in the left ventricles ($p = 0.025$) and a decrease tending towards significance ($p = 0.098$) in the right ventricles (Table 8).

**Table 8. Summary of Angiogenic & Vasculogenic Mediators in Adult Cardiac Samples: Real-Time PCR Results.**

| | | Left Ventricle | | | Right Ventricle | | |
|---|---|---|---|---|---|---|---|
| | | Control | Treatment | p value | Control | Treatment | p value |
| 9 Month | Ang1 | 0.04 ± 0.01 | 0.07 ± 0.03 | 0.420 | 0.017 ± 0.004 | 0.020 ± 0.004 | 0.625 |
| | Ang2 | 0.01 ± 0.003 | 0.02 ± 0.01 | 0.482 | 1.58 ± 0.13 | 1.44 ± 0.16 | 0.530 |
| | Tie2 | 0.92 ± 0.45 | 0.41 ± 0.17 | 0.331 | 31.07 ± 4.95 | 28.10 ± 0.63 | 0.584 |
| | VEGF | 9.67 ± 1.69 | 18.46 ± 6.26 | 0.212 | 1.0E-4 ± 1.8E-5 | 1.0E-4 ± 2.1E-5 | 0.715 |
| | VEGFR1 | 0.13 ± 0.01 | 0.16 ± 0.01 | 0.117 | 0.44 ± 0.04 | 0.43 ± 0.04 | 0.911 |
| | VEGFR2 | 74.03 ± 2.99 | 95.7 ± 10.5 | 0.059 | 0.067 ± 0.007 | 0.066 ± 0.006 | 0.949 |
| 21 Month | Ang1 | 0.033 ± 0.004 | 0.016 ± 0.005 | 0.025 | 0.047 ± 0.002 | 0.035 ± 0.006 | 0.098 |
| | Ang2 | 0.007 ± 0.001 | 0.005 ± 0.001 | 0.329 | 0.007 ± 0.001 | 0.005 ± 0.001 | 0.197 |
| | Tie2 | 1.77 ± 0.31 | 1.13 ± 0.40 | 0.242 | 10.115 ± 1.115 | 10.69 ± 0.77 | 0.690 |
| | VEGF | 4.70 ± 0.91 | 3.14 ± 0.47 | 0.177 | 6.60 ± 0.23 | 6.07 ± 0.24 | 0.186 |
| | VEGFR1 | 0.11 ± 0.01 | 0.18 ± 0.07 | 0.446 | 0.924 ± 0.050 | 1.01 ± 0.05 | 0.270 |
| | VEGFR2 | 50.93 ± 6.58 | 43.44 ± 11.61 | 0.603 | 1.677 ± 0.046 | 1.62 ± 0.05 | 0.393 |

Values are starting quantity of gene (in pg) normalized to starting quantity of RNA Polymerase II (in pg). Values reported as mean ± sem.

No differences were detected in protein concentration for any of the angiogenic and vasculogenic mediators measured by Western immunoblot (Table 9). In the 21 month right ventricle, Tie2 was not quantifiable in a reproducible, reliable manner and therefore no results are reported here. Representative blots of all proteins are shown in Attachment 1.

**Table 9. Summary of Angiogenic & Vasculogenic Mediators in Adult Cardiac Samples: Western Immunoblot Results.**

| | | Left Ventricle | | | Right Ventricle | | |
|---|---|---|---|---|---|---|---|
| | | Control | Treatment | p value | Control | Treatment | p value |
| 9 Month | Ang1 | 1.54 ± 0.35 | 1.72 ± 0.25 | 0.700 | 0.89 ± 0.25 | 1.53 ± 0.28 | 0.127 |
| | Ang2 | 4.16 ± 1.28 | 6.68 ± 2.00 | 0.291 | 2.95 ± 0.82 | 5.00 ± 1.76 | 0.271 |
| | Tie2 | 1.04 ± 0.21 | 1.36 ± 0.22 | 0.331 | 1.07 ± 0.11 | 1.18 ± 0.15 | 0.574 |
| 21 Month | Ang1 | 0.82 ± 0.15 | 0.96 ± 0.16 | 0.528 | 1.37 ± 0.42 | 1.72 ± 0.58 | 0.630 |
| | Ang2 | 1.59 ± 0.76 | 0.96 ± 0.21 | 0.458 | 1.54 ± 0.78 | 0.85 ± 0.14 | 0.424 |
| | Tie2 | 3.68 ± 0.96 | 2.63 ± 0.57 | 0.375 | Not Quantifiable | | |

Values reported as mean ± sem. 50 μg total protein loaded per well. Wells normalized to a common control run on each gel.

In the kidney, fewer changes in the angiogenic and vasculogenic mediators were seen. As in the heart, no changes where detected until Day 90, where the mRNA concentration of VEGF in the fetal kidney treatment group was significantly reduced ($p = 0.044$) (Table 10). This significance disappeared by 9 months of age and by 21 months of age, a significant decrease ($p = 0.024$) in the mRNA concentration of VEGF R2 in the treatment group was found (Table 10).

**Table 10. Summary of Angiogenic & Vasculogenic Mediators in Renal Samples: Real-Time PCR Results.**

| | | Control | Treatment | p value |
|---|---|---|---|---|
| Day 65 | Ang1 | 0.870 ± 0.055 | 0.841 ± 0.051 | 0.711 |
| | Ang2 | 0.0005 ± 0.0003 | 0.0013 ± 0.0003 | 0.119 |
| | Tie2 | 0.279 ± 0.064 | 0.305 ± 0.036 | 0.732 |
| | VEGF | 0.038 ± 0.006 | 0.028 ± 0.004 | 0.181 |
| | VEGFR1 | 0.0075 ± 0.0006 | 0.0067 ± 0.0007 | 0.411 |
| | VEGFR2 | 0.223 ± 0.0094 | 0.226 ± 0.0096 | 0.818 |
| Day 90 | Ang1 | 2.402 ± 0.128 | 2.627 ± 0.219 | 0.433 |
| | Ang2 | 0.003 ± 0.0004 | 0.0035 ± 0.0005 | 0.821 |
| | Tie2 | 1.094 ± 0.269 | 0.860 ± 0.258 | 0.544 |
| | VEGF | 0.156 ± 0.0177 | 0.099 ± 0.0178 | 0.044 |
| | VEGFR1 | 0.0058 ± 0.0016 | 0.0059 ± 0.0010 | 0.965 |
| | VEGFR2 | 1.966 ± 0.258 | 1.414 ± 0.234 | 0.144 |
| 9 Month | Ang1 | 7.363 ± 1.509 | 8.007 ± 0.478 | 0.694 |
| | Ang2 | 0.0043 ± 0.0011 | 0.0070 ± 0.0023 | 0.302 |
| | Tie2 | 0.0323 ± 0.0066 | 0.0317 ± 0.0083 | 0.954 |
| | VEGF | 0.0031 ± 0.0007 | 0.0041 ± 0.0013 | 0.551 |
| | VEGFR1 | 0.0008 ± 0.0002 | 0.0004 ±0.0001 | 0.128 |
| | VEGFR2 | 0.052 ± 0.0116 | 0.079 ± 0.0115 | 0.142 |
| 21 Month | Ang1 | 7.228 ± 1.045 | 6.486 ± 1.134 | 0.638 |
| | Ang2 | 0.001 ± 0.0003 | 0.002 ± 0.0003 | 0.879 |
| | Tie2 | 0.334 ± 0.043 | 0.373 ± 0.028 | 0.475 |
| | VEGF | 0.0034 ± 0.0007 | 0.0028 ± 0.0005 | 0.462 |
| | VEGFR1 | 0.633 ± 0.116 | 0.713 ± 0.157 | 0.689 |
| | VEGFR2 | 6.961 ± 0.650 | 4.454 ± 0.650 | 0.024 |

Values are starting quantity of gene (in pg) normalized to starting quantity of ribosomal protein s15 (in pg). Values reported as mean ± sem.

As in the heart tissues, no differences were detected in the renal protein concentration for any of the angiogenic and vasculogenic mediators measured by Western immunoblot, although Ang2 showed a slight trend down ($p = 0.106$) in the treatment group (Table 11). In both treatment groups, Tie2 was not quantifiable in a reproducible, reliable manner and therefore no results are reported here.

**Table 11. Summary of Angiogenic & Vasculogenic Mediators in Adult Renal Samples: Western Immunoblot Results.**

| | | Control | Treatment | p value |
|---|---|---|---|---|
| 9 Month | Ang1 | 0.84 ± 0.10 | 1.16 ±0.41 | 0.396 |
| | Ang2 | 0.90 ± 0.03 | 0.85 ± 0.20 | 0.782 |
| | Tie2 | | Not Quantifiable | |
| 21 Month | Ang1 | 1.25 ± 0.18 | 1.11 ± 0.29 | 0.675 |
| | Ang2 | 0.91 ± 0.16 | 0.56 ± 0.11 | 0.106 |
| | Tie2 | | Not Quantifiable | |

Values are mean Corrected Arbitrary Units ± sem. 50 µg total protein loaded per well. Wells normalized to a common control run on each gel.

## Hemodynamic Mediators

As with the angiogenic and vasculogenic mediators, no changes in mRNA concentration were detected at fetal Day 65 in any of the hemodynamic mediators. By fetal Day 90, changes in the cardiac mRNA concentration of AT2 tended to be reduced ($p = 0.102$) in the treatment group but then disappeared with age (Table 12).

**Table 12. Summary of Hemodynamic Mediators in Fetal Cardiac Samples: Real-Time PCR Results.**

| | | Control | Treatment | p value |
|---|---|---|---|---|
| **Day 65** | **AT1** | 0.069 ± 0.0059 | 0.0903 ± 0.0153 | 0.215 |
| | **AT2** | 0.0682 ± 0.007 | 0.0979 ± 0.0171 | 0.135 |
| | **eNOS** | 0.0722 ± 0.0047 | 0.0868 ± 0.0131 | 0.313 |
| **Day 90** | **AT1** | 0.291 ± 0.094 | 0.432 ± 0.09 | 0.314 |
| | **AT2** | 0.047 ± 0.014 | 0.021 ± 0.007 | 0.102 |
| | **eNOS** | 0.157 ± 0.015 | 0.150 ± 0.0147 | 0.966 |

Values are starting quantity of gene (in pg) normalized to starting quantity of RNA Polymerase II (in pg). Values reported as mean ± sem.

While no changes were detected in the left ventricle at any age for AT1, a significant decrease ($p = 0.004$) was seen in the right ventricle of the treatment group at 21 months of age (Table 13). The most drastic changes detected in the hemodynamic mediators started at 9 months of age and were found in eNOS mRNA concentrations. In the left ventricle, eNOS mRNA concentration tended to be elevated ($p = 0.084$) in the treatment group at 9 months of age, but by 21 months of age, it tended to be reduced ($p = 0.052$) in the treatment group (Table 13). In the right ventricle, eNOS mRNA concentration was significantly reduced ($p = 0.019$) in the treatment group at 9 months of age and even more significantly reduced ($p < 0.001$) by 21 months of age (Table 13).

**Table 13. Summary of Hemodynamic Mediators in Adult Cardiac Samples: Real-Time PCR Results.**

| | | Left Ventricle | | | Right Ventricle | | |
|---|---|---|---|---|---|---|---|
| | | Control | Treatment | p value | Control | Treatment | p value |
| 9 Month | AT1 | 0.135 ± 0.0013 | 0.133 ± 0.0006 | 0.240 | 6.106 ± 0.595 | 5.100 ± 0.096 | 0.171 |
| | AT2 | 0.006 ± 0.0003 | 0.005 ± 0.0004 | 0.221 | 14.421 ± 1.036 | 13.390 ± 0.724 | 0.461 |
| | eNOS | 0.188 ± 0.006 | 0.269 ± 0.041 | 0.084 | 0.040 ± 0.003 | 0.027 ± 0.002 | 0.019 |
| 21 Month | AT1 | 0.150 ± 0.034 | 0.114 ± 0.034 | 0.467 | 5.893 ± 0.329 | 4.521 ± 0.215 | 0.004 |
| | AT2 | 0.019 ± 0.006 | 0.015 ± 0.004 | 0.535 | 2.883 ± 0.157 | 2.660 ± 0.166 | 0.345 |
| | eNOS | 0.127 ± 0.012 | 0.088 ± 0.013 | 0.052 | 0.168 ± 0.010 | 0.087 ± 0.008 | < 0.001 |

Values are starting quantity of gene (in pg) normalized to starting quantity of RNA Polymerase II (in pg). Values reported as mean ± sem.

Protein data also showed a decrease ($p = 0.032$) in eNOS in the left ventricle at 21 months of age (Table 14). Interestingly, protein data for eNOS in the right ventricle indicated an increase ($p = 0.052$) in eNOS in the treatment group at 21 months of age (Table 14), indicating that there may be effects within the pulmonary circulation that are differentially impacting the right side of the heart compared to the systemic effects that would primarily impact the left side of the heart. In both the left and right ventricles, AT2 was not quantifiable in a reproducible, reliable manner and therefore no results are reported here. In addition, due to the small amount of fetal tissue available, protein data was not collected from fetal Day 65 or fetal Day 90 cardiac samples.

**Table 14. Summary of Hemodynamic Mediators in Adult Cardiac Samples: Western Immunoblot Results.**

| | | Left Ventricle | | | Right Ventricle | | |
|---|---|---|---|---|---|---|---|
| | | Control | Treatment | p value | Control | Treatment | p value |
| 9 Month | AT1 | 1.22 ± 0.16 | 1.24 ± 0.17 | 0.955 | 1.31 ± 0.57 | 2.16 ± 0.46 | 0.300 |
| | AT2 | Not Quantifiable | | | Not Quantifiable | | |
| | eNOS | 0.92 ± 0.13 | 0.52 ± 0.20 | 0.119 | 0.92 ± 0.26 | 1.56 ± 0.62 | 0.313 |
| 21 Month | AT1 | 1.26 ± 0.11 | 1.57 ± 0.26 | 0.274 | 1.52 ± 0.15 | 1.27 ± 0.13 | 0.227 |
| | AT2 | Not Quantifiable | | | Not Quantifiable | | |
| | eNOS | 1.14 ± 0.13 | 0.63 ± 0.18 | 0.032 | 2.34 ± 0.43 | 3.80 ± 0.56 | 0.052 |

Values are mean Corrected Arbitrary Units ± sem. 50 μg total protein loaded per well. Wells normalized to a common control run on each gel.

In the kidney, fewer changes in the hemodynamic mediators were detected, again with no significant changes seen at fetal Day 65. At fetal Day 90, changes in eNOS mRNA concentration were found, with an increase ($p = 0.059$) in the treatment group.

While the mRNA concentration was still elevated, the significance was lost by 9 months of age (Table 15).

**Table 15. Summary of Hemodynamic Mediators in Renal Samples: Real-Time PCR Results.**

| | | Control | Treatment | p value |
|---|---|---|---|---|
| **Day 65** | **AT1** | 0.084 ± 0.007 | 0.112 ± 0.018 | 0.215 |
| | **AT2** | 0.106 ± 0.021 | 0.114 ± 0.008 | 0.135 |
| | **eNOS** | 0.026 ± 0.004 | 0.036 ± 0.005 | 0.313 |
| **Day 90** | **AT1** | 0.370 ± 0.030 | 0.401 ± 0.049 | 0.590 |
| | **AT2** | 2.478 ± 0.423 | 2.215 ± 0.275 | 0.612 |
| | **eNOS** | 0.081 ± 0.025 | 0.174 ± 0.034 | 0.059 |
| **9 Month** | **AT1** | 0.364 ± 0.035 | 0.547 ± 0.996 | 0.121 |
| | **AT2** | 1.380 ± 0.315 | 1.807 ± 0.200 | 0.296 |
| | **eNOS** | 0.0069 ± 0.0022 | 0.0126 ± 0.0029 | 0.153 |
| **21 Month** | **AT1** | 0.244 ± 0.048 | 0.300 ± 0.041 | 0.396 |
| | **AT2** | 42.122 ± 2.373 | 42.572 ± 4.670 | 0.930 |
| | **eNOS** | 0.0045 ± 0.0009 | 0.0051 ± 0.0007 | 0.553 |

Values are starting quantity of gene (in pg) normalized to starting quantity of ribosomal protein s15 (in pg). Values reported as mean ± sem.

As with the cardiac tissues above, protein data was not collected from fetal Day 65 or fetal Day 90 renal samples due to the small amount of fetal tissue available. In adult renal tissues, the eNOS protein concentration at 9 months of age was significantly elevated ($p = 0.027$) in response to prenatal T-treatment, but by 21 months of age the significance was lost despite still being elevated in the treatment group (Table 16). As was the case in the left and right ventricles, AT2 protein in the adult kidney was not quantifiable in a reproducible, reliable manner and therefore no results are reported here.

**Table 16. Summary of Hemodynamic Mediators in Adult Renal Samples: Western Immunoblot Results.**

| | | Control | Treatment | p value |
|---|---|---|---|---|
| **9 Month** | **AT1** | 1.48 ± 0.39 | 1.20 ± 0.23 | 0.606 |
| | **AT2** | | Not Quantifiable | |
| | **eNOS** | 1.22 ± 0.15 | 1.95 ± 0.26 | 0.027 |
| **21 Month** | **AT1** | 2.45 ± 0.62 | 1.67 ± 0.29 | 0.320 |
| | **AT2** | | Not Quantifiable | |
| | **eNOS** | 1.79 ± 0.20 | 2.05 ± 0.12 | 0.337 |

Values are mean Corrected Arbitrary Units ± sem. 50 µg total protein loaded per well. Wells normalized to a common control run on each gel.

**Discussion**

Exposure to excess prenatal androgens has been previously shown to cause growth restriction, alter sexual development and function, and cause the onset of symptoms that closely resemble those seen in women with polycystic ovary syndrome, including the metabolic syndrome. Previously reported differences in cardiac and kidney weights of prenatally testosterone treated female offspring at 21 months of age were suggestive of the development of systemic hypertension (Padmanabhan *et al.* 2004). Despite lacking physiological measures to correlate the differences in cardiac and kidney weights with, the selective increase in left ventricle + septum and kidney weights suggested that the prenatal androgen exposure altered normal cardiovascular function as the animals aged. This observation led to the current study to evaluate whether prenatal androgen excess influences cardiovascular development and can lead to adulthood disease by altering expression of key mediators in the heart and kidneys, as well as if it also alters metabolic mediators important in glucose regulation. During the present study, King *et al.* showed that prenatal exposure to the same level and timing of excess testosterone as the current study significantly increased arterial pressure and resulted in mild hypertension in 2 year old adult female sheep (King *et al.* 2007). The results of King's study further demonstrate that the sheep model of PCOS shares common risk factors seen in women with PCOS and supports the current hypothesis that prenatal testosterone exposure leads to cardiovascular and metabolic changes.

A closer look at the data from the earlier study showed a loss of significance at 21 months when the left ventricle + septum and kidney weights were normalized to body weight. However, when normalized to body weight at 9 months of age, a significant

increase was seen in the kidney weight of the treated offspring. This may be due to an increase in nephron size as a result of the metabolic demand placed on the postnatal kidney as a result of catch-up growth experienced after exposure to a suboptimal uterine environment. Unlike the fetal kidney, the postnatal kidney is unable to increase nephron number in response to growth-related demands and attempts to compensate by enlarging existing nephron units through hypertrophy (Bagby 2007). While there was also a significant decrease in the normalized total heart weight of the treatment group at 9 months, there was no difference in the normalized weight of the left side of the heart, or in the right ventricle when separated. This suggests that the difference in total heart weight may have been due to the inclusion of the atria. The increase in the kidney weight at 9 months of age may be the first indication that a change in systemic blood pressure has occurred and the enlarged kidneys are an attempt to regulate it. This is supported by numerous experimental IUGR models, across species, that have reproducibly shown reduced nephron number at birth and subsequent elevated blood pressure (Bagby 2007). Determining what structural changes, if any, have occurred in the heart and kidneys of the treatment group would provide further insight into what is occurring that may be impacting cardiovascular function.

No differences in fetal cardiac or renal weights were seen at either Day 65 or Day 90. At fetal Day 65, the normalized kidney weight in the treatment group was significantly reduced. By 65 dGA, the fetal kidney is in the middle of development, with nephrogenesis being complete by 130 days of gestation (Moritz *et al.* 2003). In the sheep, as in humans, nephrogenesis is completed by the time of birth. Any stressor during this period of renal development has the potential to drastically alter renal function

throughout life. In offspring that have experienced intrauterine growth restriction, a permanent deficit in nephron number has been shown to occur (Merlet-Benichou 1999). While a reduction in nephron number may cause the development of hypertension, defects in renal vascular development may also occur and be involved in the etiology of hypertension later in life (Merlet-Benichou 1999). Studies in sheep have shown that exposure to high levels of dexamethasone for only 2 days around 27 days of gestation results in the development of hypertension by 3 – 4 months of age (Dodic *et al.* 1998). At 7 years of age, the dexamethasone exposed offspring had a ~ 60% reduction in glomeruli and nephron number compared to the control animals, with no changes in sclerosis index or kidney weight (Moritz *et al.* 2003). In addition to reducing nephrogenesis, exposure to dexamethasone between 60 – 75 dGA also causes accelerated maturation of the ovine fetal kidney (Moritz *et al.* 2003; Wintour *et al.* 1994). The decreased nephron number seen in these animals in adulthood most likely occurred due to a reduction in nephron number during fetal development. As intrauterine programming by glucocorticoid and testosterone excess share many common phenotypic outcomes (King *et al.* 2007; Fowden *et al.* 2006), it seems plausible that exposure to excess androgens in the current study resulted in a similar reduction in nephron number. The difference in kidney weight seen at fetal Day 65 may be indicative of this in the treatment group. The loss of significance in kidney weight by fetal Day 90 may be a result of hypertrophy to compensate for the reduced nephron number. By 9 months of age, the increase in kidney weight may simply be due to the fact that the ewes in the treatment group were larger than the controls. A follow-on study looking at histological sections of the kidneys at the current time points, plus an additional fetal time point around 130 –

140 dGA would help determine the impact androgen excess has on renal development and function.

To determine the cardiovascular effects of prenatal exposure to excess testosterone, protein and mRNA concentrations of angiogenic/vasculogenic and hemodynamic mediators were evaluated from fetal total hearts, fetal kidney, adult left ventricle, adult right ventricle, and adult kidney. With the angiogenic/vasculogenic mediators angiopoietin 1 (Ang1), angiopoietin 2 (Ang2), their receptor tunica interna endothelial cell kinase-2 (Tie2), vascular endothelial growth factor (VEGF), VEGF receptor 1 (VEGF R1), and VEGF receptor 2 (VEGF R2), no differences were seen at either of the fetal time points studied. Few studies evaluating fetal angiogenic factors are available, with even less available on VEGF-related growth factors in IUGR complicated pregnancies (Wallner *et al.* 2007). While no significance was seen in the cardiac mRNA concentrations for any of these mediators, it was interesting to note a reversal in the mRNA concentrations of VEGF, VEGF R1, and VEGF R2 over the course of fetal development. At fetal Day 90, the mRNA concentrations of VEGF, VEGF R1, and VEGF R2 were lower in the treatment group compared to controls, whereas earlier at fetal Day 65, they were elevated in the treatment group. Again, although not statistically different, it may indicate that something is occurring within the cardiovascular system. In comparing serum concentrations of maternal and fetal angiogenic growth factors in IUGR, Wallner *et al.* found that soluble VEGF R1 was increased, soluble VEGF R2 decreased, and no change in VEGF in serum collected from the umbilical vein (Wallner *et al.* 2007). Differences from the current study may represent the fact that collection of umbilical blood represents whole body concentrations of the various angiogenic markers

and may not necessarily be indicative of tissue-specific changes that are occurring. By 9 months of age, VEGF R2 mRNA concentration was increased in the left ventricle of the treatment group. This increase in VEGF R2 may be a cardioprotective attempt by the left ventricle to aid cardiomyocyte survival via the pathways described in Figure 3.

Hypertrophy of cardiac myocytes is an adaptive response in the damaged heart and initially acts as a compensatory mechanism to preserve cardiac function (Levy 2005). The non-significant increases seen in Ang1, Ang2, VEGF, and VEGF R1 mRNA concentrations in the treatment group may also indicate the early stages of cardioprotection. By 21 months of age, all the angiogenic/vasculogenic mediators in the left ventricle, except VEGF R1 and Tie2, have reversed and are now reduced in the treatment group. At 21 months, Ang1 is significantly reduced in the treatment group, and may be the start of cardiac dysfunction within the cardiomyocytes of the left ventricle. The decrease in eNOS mRNA and protein concentrations also seen at 21 months may further diminish the pro-angiogenic effects of Ang1, as the Ang1-induced angiogenic actions have been shown to require eNOS (Babaei *et al.* 2003). In the right ventricle, the changes were less consistent and may be due to there being less of an impact on the pulmonary circulation, and therefore the right side of the heart, compared to the left ventricle dealing with potential changes in the systemic circulation.

As with the angiogenic/vasculogenic mediators, no significant differences were detected at either fetal time point for the hemodynamic mediators AT1, AT2, or eNOS in the heart. Angiotensin II (Ang II), the active component of the renin-angiotensin system (RAS), is important in the regulation of blood pressure. All components of the RAS have been detected in the vasculature and Ang II is known to play a pathogenic role in the

development of cardiac hypertrophy and hypertension (Levy 2005). Signaling through the AT1 and AT2 receptor subtypes determines the outcome of Ang II interaction with target tissues. By fetal Day 90, AT2 mRNA concentration tended to be reduced in the treatment group compared to controls. AT2, which is highly expressed in fetal tissues, is expected to decrease postnatally. However, the decrease seen in the treatment group may be accelerated in response to excess androgen exposure and if so, the cardioprotective actions of AT2 signaling may be reduced. The lack of differences in AT1 at any time point is contrary to the findings of Samuelsson *et al.* who found an increase in both AT1 and AT2 levels in rats made hyperinsulinemic (Samuelsson *et al.* 2006). In addition, while no differences were seen in protein concentrations, the current study found a significant decrease in AT1 mRNA concentration in 21 month right ventricle and a decrease in AT2 mRNA concentration in fetal Day 90 heart. The differences between mRNA and protein concentrations among the various studies may reflect differences in species, signaling cascades, or post-translational modification. In the sheep model of PCOS, hyperinsulinemia that occurs following exposure to excess fetal androgen may also reflect alterations in feedback mechanisms that regulate the interaction between Ang II signaling via AT1 receptors and insulin signaling via the insulin receptor. At fetal Day 90, the decrease seen in cardiac AT2 mRNA concentration, and presumably a reduction in AT2 concentration, would potentially favor signaling via the AT1 pathway and allow cardiac remodeling to occur. As mentioned earlier, AT2 is normally highly expressed in fetal tissue, so the decrease seen at fetal Day 90 may be indicative of overall cardiovascular changes starting in the fetal heart.

Recabarren *et al.* have shown that female offspring from testosterone treated ewes have higher basal insulin levels and impaired sensitivity (Recabarren *et al.* 2005), presumably through the insulin receptor. The hyperinsulinemia in this sheep model may result in increased interaction between insulin and AT1 receptors that subsequently allows cardiac remodeling. The decrease in AT1 mRNA concentration in 21 month right ventricle may result from a negative feedback mechanism in response to this increased interaction. The decreased AT1 signaling would also reduce the interaction that Ang II has, via the AT1 receptor, with insulin signaling that will be discussed later.

In rats, maternal protein restriction is known to lead to fetal growth restriction, increase post-natal blood pressure, alter renal structure, as well as other changes seen with PCOS and the metabolic syndrome (Sahajpal and Ashton 2005; McMullen *et al.* 2004; Samuelsson *et al.* 2006). In 4 week old offspring of rats exposed to maternal low protein diets, Sahajpal *et al.* found that renal AT2 was decreased by 35% (Sahajpal and Ashton 2005). This is consistent with other studies of protein restriction in rats that have shown significantly lower renal AT2, higher blood pressure, and reduced nephron number (McMullen *et al.* 2004). In contrast, Zohdi *et al.* found no differences in renal AT1 or AT2 mRNA in IUGR sheep at gestation day 130, despite having significantly reduced kidney weights and nephron numbers (Zohdi *et al.* 2007). In contrast to Zohdi and the current study, Rüster *et al.* found a significant increase in renal AT1 and AT2 concentrations in IUGR newborn piglets (Ruster *et al.* 2006). At fetal Day 90, AT1 mRNA results agree with those from Rüster's newborn piglets, and while the significance is lost after birth, renal AT1 mRNA concentration is still elevated out to 21 months of age. The differences seen in mRNA concentrations between the studies, including the

current study, may be due to species differences in nephrogenesis. In humans, sheep, and pigs, nephrogenesis is completed prior to birth (Zohdi *et al.* 2007), while in the rat it continues until after birth (Ruster *et al.* 2006). Other differences between the studies, such as using whole tissue homogenates vs. specific renal zones, timing of collection, and timing and type of fetal insult, may also explain differences. The structural changes, with or without alterations in the renal RAS, that take place during fetal development may not become evident from a disease standpoint until later in life.

Potential consequences of the changes in Ang II regulation and signaling include the loss of nitric oxide production due to decreased activation of eNOS, ventricular thickening, and cardiac hypertrophy (Figure 5). By 9 months of age, changes in eNOS mRNA concentration are noticeable, starting with a tendency to be increased in the left ventricle of the treatment group. This increase in eNOS mRNA concentration may be another mechanism whereby the left ventricle is responding to downstream changes as eNOS has cardioprotective actions, including working to prevent cardiac hypertrophy. As with many of the other mediators studied, there was a reversal in concentration by the time the offspring reached 21 months of age. At this time point, eNOS mRNA concentration was reduced in the treatment group compared to controls. In addition, the protein concentration of eNOS was significantly reduced. Kobayashi *et al.* showed a decrease in left ventricle eNOS mRNA and protein concentrations in rats who experienced endothelial damage, myocardial remodeling, and congestive heart failure (Kobayashi *et al.* 1999). The shift seen in eNOS mRNA and protein concentrations from 9 months of age to 21 months of age is suggestive of a loss of cardioprotection that allows the left ventricle to remodel in response to systemic hypertension.

In the right ventricle, changes were also seen in eNOS mRNA concentrations. At 9 months of age, eNOS mRNA concentration is significantly reduced in the treatment group and becomes even more significantly reduced at 21 months of age. Interestingly, while there was a significant reduction in eNOS mRNA concentration at 21 months, eNOS concentration was nearly significantly increased in the right ventricle at 21 months of age. Since nitric oxide plays a role in limiting cardiac remodeling and providing cardiovascular protection (Rastaldo *et al.* 2007), it may be that the difference between the mRNA and protein concentrations is the result of posttranslational events occurring within the right ventricle to extend the activity of eNOS. While not significant, right ventricle weight, normalized to body weight, was reversed between 9 and 21 months, and at 21 months of age, normalized right ventricle weight is increased in the treatment group. Taken together, the shift in right ventricle weight and reduced eNOS mRNA concentration may be indicative of changes that are beginning to occur within the pulmonary circulation as well. In addition to eNOS mRNA, AT1 mRNA concentration was also significantly reduced in the right ventricle of the treatment group. A decrease in AT1 signaling may be an attempt by the myocardium to improve function by shifting away from AT1 induced hypertrophy, stimulate eNOS activity, and prevent right ventricular failure. Measurement of pulmonary pressures would help provide a more thorough understanding of what is occurring within the pulmonary circulation and the right ventricle.

In the kidney, no significant differences were seen in any of the angiogenic/vasculogenic mediators at fetal Day 65. At fetal Day 90, VEGF mRNA concentration was significantly lower in the treatment group compared to controls.

While not significantly different at fetal Day 65, VEGF mRNA concentration was reduced in the treatment group as well. This reduction in VEGF mRNA concentration during fetal development may indicate altered renal development is occurring, especially if the non-significant reduction of VEGF R2 mRNA concentration and altered fetal kidney weights are considered. As in the heart, VEGF R2 mRNA concentration in the treatment group is reversed at 21 months of age when compared to the concentration at 9 months of age in the left ventricle. However, at 21 months of age, the reduction of VEGF R2 mRNA concentration seen in the treatment group is now significant. There was also a decrease in the protein concentration of Ang2 which could affect branching angiogenesis within the kidney, which would lead to reduced renal vascularization and potentially renal hypertension. Taken together, these changes suggest that the kidney is beginning to fail to maintain renal tissue function and survival due to the loss of VEGF signaling as shown in Figure 3.

Hemodynamically, renal eNOS mRNA concentration was increased at fetal Day 90. Manning *et al.* reported that several studies in rats have shown that inhibition of NO synthesis leads to both short-term and long-term renal vasoconstriction (Manning, Jr. and Hu 1994; Manning, Jr. *et al.* 1994). Long-term increases in renal blood pressure can lead to increases in arterial pressure. The increase in eNOS mRNA concentration at fetal Day 90 may therefore provide some protection of renal blood pressure by reducing vascular resistance. While still elevated in the treatment group at 9 months of age, eNOS mRNA concentration was no longer significant. However, at 9 months of age eNOS concentration was significantly elevated in the treatment group. Again, this may be in response to increased systemic blood pressure and an attempt to compensate. By 21

months of age, both eNOS mRNA and protein concentrations are elevated in the treatment group, but not significantly. The age differential in eNOS between the heart and kidney indicates that a shift in vasoregulation is occurring. This shift within the kidney, starting at fetal Day 90, may be driving the changes that are seen in the other mediators at 21 months of age.

When taken as a whole, the data supports the development of systemic hypertension as offspring exposed to excess prenatal androgen age. While direct measurements of blood pressure are missing in this study, King *et al.* reported mild hypertension at 2 years of age in female sheep exposed to the same treatment as the current study (King *et al.* 2007). The lack of changes during early development (fetal Days 65 and 90) suggests that there is less of a direct effect of androgen excess on fetal cardiovascular development and more of an indirect effect on other aspects of the uterine environment, such as placental development. Androgen exposure may have more of a direct effect on other aspects of fetal development, such as in the hypothalamic-pituitary-gonadal axis and the reproductive effects seen with this ovine model of PCOS as previously described by numerous researchers. The lack of early changes suggests that the effects seen later in the cardiovascular system may be a result of fetal programming occurring as a result of placental insufficiency. An additional fetal time point around 130 – 140 dGA may have helped detect changes that appear later in fetal development.

# CHAPTER IV

## Alterations in Cardiac and Renal IRβ, GLUT1, and GLUT4 in Response to Fetal Androgen Exposure

### Introduction

During fetal development, a complex interaction between numerous growth factors, receptors, and signaling pathways takes place to establish the regulation of the various hormonal and metabolic functions necessary for normal development, both during the fetal period as well as later in life. Alterations in the fetal environment during this time can modify normal gene expression, protein concentrations, metabolic cascades, and other physiologic functions. In addition, the set point and sensitivity of these systems can also be altered (Fowden *et al.* 2005). Often, these fetal changes are in response to an environment where nutrient supply to the developing fetus is restricted. The result of this fetal programming is typically a survival adaptation for the developing fetus, but often leads to complications later in adulthood. The negative consequences that appear later in life are usually due to the postnatal environment no longer posing a nutritional challenge to survival. In the presence of excess nutrients, the reprogrammed hormonal and metabolic pathways respond incorrectly and the health of the individual suffers.

In hearts from a non-obese rat model of type II diabetes, Desrois *et al.* found a significant loss of the insulin receptor β (IRβ), suggesting that the loss or down-regulation of IRβ underlies the development of insulin resistance (Desrois *et al.* 2004).

They also found significant loss of glucose transporter 4 (GLUT4) and insulin receptor substrate 1 (IRS-1). The loss of these proteins reduces insulin sensitivity by interfering with downstream signaling via various second messenger pathways and therefore alters metabolism (Desrois *et al.* 2004). The loss of the IRβ and IRS-1 proteins results in reduced insulin signaling, while the reduced GLUT4 potentially impairs insulin-stimulated glucose uptake in the heart. If also occurring in skeletal muscle, this would further lead to peripheral insulin resistance.

In addition to regulating the metabolic effects of insulin, normal insulin signaling is also critical for regulating cardiac growth. Abnormal insulin regulation, such as in the metabolic syndrome, is linked with cardiovascular pathologies. Left ventricular hypertrophy has been shown to be associated with hyperinsulinemia and impaired insulin-mediated glucose uptake in hypertensive patients (Paolisso *et al.* 1995; Sharp and Williams 1992) but the relationship between the hypertrophy and elevated blood pressure only explains 25 – 30% of the variation in left ventricular mass, suggesting that other mechanisms are involved (Samuelsson *et al.* 2006; Prisant and Carr 1990). Insulin is an important cardiac growth factor and works in conjunction with other mediators to simulate cardiac and vascular development. Specifically, activation of $PI_3$ kinase ($PI_3K$) via tyrosine phosphorylation activates Akt/PKB, which is an important mediator in cardioprotection (Taegtmeyer *et al.* 2005). Activation of Akt/PKB via stimulation of IRβ plays a critical role in the induction of cardiac hypertrophy in response to hypertension by phosphorylation of numerous downstream effectors. Akt/PKB also plays an important role in regulating normal metabolic substrate utilization by phosphorylation of GLUT4, promoting its translocation to the plasma membrane and facilitating glucose uptake

(Taegtmeyer *et al.* 2005). An alteration in the normal phosphorylation of the insulin receptor, and possibly other receptors and pathways, may play a role in reduced fetal growth, hyperinsulinemia, and hyperandrogenism (Ibanez *et al.* 1998).

We hypothesized that prenatal exposure to excess testosterone from days 30 to 90 of gestation will up-regulate the mRNA and protein concentrations of angiogenic growth factors and mediators of insulin signaling and glucose metabolism in treated fetal cardiac and kidney tissues and then decrease postnatally in an age-dependent manner. To determine the metabolic effects of prenatal exposure to excess testosterone, protein and mRNA concentrations of mediators that are important in the regulation of glucose transport and metabolism were evaluated from fetal total hearts, fetal kidney, adult left ventricle, adult right ventricle, and adult kidney. As normal glucose signaling and control is critical for maintaining fetal and adult health, variations in gene expression and protein concentration may provide insight into what is occurring as we age that can help diagnosis, prevent, and treat the on-set of insulin resistance and diabetes. To study the impact on metabolic regulation and development in response to excess fetal testosterone and fetal growth restriction, the following metabolic mediators were studied: insulin receptor β (IRβ), glucose transporter 1 (GLUT1), and glucose transporter 4 (GLUT4).

**Methods & Materials**

The methods and materials for the study of the metabolic mediators in this chapter are the same as those described in the previous chapter for the cardiovascular mediators. The following are specific changes that apply for the analysis of glucose transporter 1 (GLUT1), glucose transporter 4 (GLUT4), and insulin receptor β (IRβ).

### *Primer Design*

Gene-specific forward and reverse primers were designed for the following genes to be analyzed via real-time PCR: glucose transporter 1 (GLUT1), glucose transporter 4 (GLUT4), and insulin receptor β (IRβ). All primers were designed from published gene sequences and were designed to span at least one intron to distinguish genomic contamination. Table 17 lists the primer sequence, product size, annealing temperature, and accession number for the genes of the metabolic mediators validated for real-time PCR. Complete sequences for each cDNA are listed in Attachment 2.

**Table 17. Summary of Ovine Primers used in Real-Time PCR Analysis of Metabolic Mediators.**

| Gene | Forward Primer | Reverse Primer | Length (bp) | Temp (°C) | Accession Number |
|---|---|---|---|---|---|
| **GLUT1** | gacagggaggagcaagccaaa | tagggtgaagccagggatgtg | 380 | 61 | U89029 |
| **GLUT4** | tggccttctttgaaattggccctg | atccgccacatactggaaacccat | 155 | 61 | AT949177 |
| **IRβ** | cagctgagctagaagccaac | ccatttcagcaagatcttgtc | 415 | 57 | AY157728 |
| **s15** | atcattctgcccgagatggtg | tgctttacgggcttgtaggtg | 134 | 60 | NM_001018 |
| **Pol II** | agtccaacatgctgacggacatga | agccaagtgccggtaattgacgta | 332 | 60 | CD288457 |

### *Western Blot Analysis*

Membranes were incubated with primary antibodies (Santa Cruz Biotechnology) against insulin receptor β (sc-711 HRP-conjugated, 1:900). Protein concentrations for glucose transporters 1 and 4 have yet to be determined.

### *Statistical Analysis*

Primary outcome measures were mRNA concentration and protein concentration. Statistical differences between prenatal treatments, for a given age, were determined by Students t-test with a p-value $\leq 0.05$ accepted as statistically significant. Unless otherwise noted, values were reported as mean ± sem.

## Results

### Cardiac Tissues

As with the angiogenic/vasculogenic and hemodynamic mediators discussed in the previous chapter, few changes were detected in the metabolic mediators evaluated at either of the fetal time points (Table 18). Not until fetal Day 90 are alterations in any of the metabolic mediators seen. At fetal Day 90, a significant increase ($p = 0.021$) in the mRNA concentration of insulin receptor β was seen in the cardiac tissue from prenatally T-treated fetuses (Table 18).

**Table 18. Summary of Metabolic Mediators in Fetal Cardiac Samples: Real-Time PCR Results.**

| | | Control | Treatment | p value |
|---|---|---|---|---|
| Day 65 | GLUT1 | 0.0095 ± 0.0005 | 0.0103 ± 0.0006 | 0.299 |
| | GLUT4 | 0.0074 ± 0.0006 | 0.0085 ± 0.0009 | 0.336 |
| | IRβ | 0.0093 ± 0.0005 | 0.010 ± 0.0005 | 0.279 |
| Day 90 | GLUT1 | 9.793 ± 1.669 | 9.292 ± 1.635 | 0.835 |
| | GLUT4 | 0.132 ± 0.029 | 0.227 ± 0.061 | 0.246 |
| | IRβ | 0.0029 ± 0.0007 | 0.0051 ± 0.0004 | 0.021 |

Values are starting quantity of gene (in pg) normalized to starting quantity of RNA Polymerase II (in pg). Values reported as mean ± sem.

The increase in IRβ first seen at fetal Day 90 continued out to 9 months of age in the treatment group; however, significance in the mRNA concentration (Table 19) was lost despite the presence of a significant increase ($p = 0.0094$) in IRβ concentration in the left ventricle (Table 20).

**Table 19. Summary of Metabolic Mediators in Adult Cardiac Samples: Real-Time PCR Results.**

| | | Left Ventricle | | | Right Ventricle | | |
|---|---|---|---|---|---|---|---|
| | | Control | Treatment | p value | Control | Treatment | p value |
| 9 Month | GLUT1 | 16.11 ± 2.04 | 20.52 ± 3.48 | 0.305 | 3.60 ± 0.29 | 2.99 ± 0.10 | 0.112 |
| | GLUT4 | 12.50 ± 2.60 | 12.82 ± 3.22 | 0.939 | 0.61 ± 0.06 | 0.47 ± 0.002 | 0.097 |
| | IRβ | 0.0055 ± 0.001 | 0.0064 ± 0.002 | 0.646 | 0.0020 ± 0.0002 | 0.0018 ± 0.0003 | 0.736 |
| 21 Month | GLUT1 | 7.26 ± 1.03 | 3.94 ± 0.42 | 0.013 | 1.77 ± 0.07 | 1.52 ± 0.04 | 0.006 |
| | GLUT4 | 3.24 ± 0.45 | 1.96 ± 0.65 | 0.147 | 0.39 ± 0.05 | 0.19 ± 0.01 | 0.003 |
| | IRβ | 0.014 ± 0.004 | 0.007 ± 0.001 | 0.136 | 1.78 ± 0.18 | 1.44 ± 0.15 | 0.169 |

Values are starting quantity of gene (in pg) normalized to starting quantity of RNA Polymerase II (in pg). Values reported as mean ± sem.

Interestingly, a shift in IRβ concentration was seen in the left ventricle at 21 months of age. By 21 months, IRβ concentration was significantly reduced ($p = 0.036$) (Table 20), and while not significant, the mRNA concentration was much lower in the treatment group as well (Table 19). Due to the small amount of fetal tissue available, protein data was not collected from fetal Day 65 or Day 90 cardiac samples.

**Table 20. Summary of Metabolic Mediators in Adult Cardiac Samples: Western Immunoblot Results.**

| | | Left Ventricle | | | Right Ventricle | | |
|---|---|---|---|---|---|---|---|
| | | Control | Treatment | p value | Control | Treatment | p value |
| 9 Month | IRβ | 1.18 ± 0.18 | 2.80 ± 0.63 | 0.009 | 1.75 ± 0.24 | 1.31 ± 0.08 | 0.163 |
| 21 Month | IRβ | 0.91 ± 0.09 | 0.59 ± 0.11 | 0.036 | 1.37 ± 0.15 | 1.63 ± 0.20 | 0.307 |

Values are mean Corrected Arbitrary Units ± sem. 50 µg total protein loaded per well. Wells normalized to a common control run on each gel.

In addition to changes in insulin receptor β, potential changes in glucose transport into the cardiac tissues were seen at 9 months of age. In the left ventricle, no changes were seen in either of the glucose transporters studied. However, changes were seen in GLUT4 mRNA concentrations in the right ventricle. At 9 months of age, GLUT4 mRNA concentration tended to be reduced ($p = 0.097$) in the treatment group (Table 19). This decrease was even more significant ($p = 0.003$) in the right ventricle at 21 months of age (Table 19), suggesting that the right ventricle is becoming insulin resistant with age. By 21 months of age, significant changes in GLUT1 mRNA concentration were detected in both the left ($p = 0.013$) and right ventricles ($p = 0.006$) (Table 19).

## Renal Tissues

As with the angiogenic/vasculogenic and hemodynamic mediators discussed in the previous chapter, few changes were detected in the metabolic mediators evaluated at any of the time points studied (Table 21). The only significant change occurred at 9 months of age where a significant increase ($p = 0.047$) in GLUT4 mRNA concentration was seen in the treatment group (Table 21).

**Table 21. Summary of Metabolic Mediators in Renal Samples: Real-Time PCR Results.**

| | | Control | Treatment | p value |
|---|---|---|---|---|
| Day 65 | GLUT1 | 0.095 ± 0.0081 | 0.084 ± 0.0078 | 0.377 |
| | GLUT4 | 0.0081 ± 0.0005 | 0.0089 ± 0.0006 | 0.383 |
| | IRβ | 0.0061 ± 0.0009 | 0.0047 ± 0.0007 | 0.257 |
| Day 90 | GLUT1 | 0.344 ± 0.032 | 0.317 ± 0.017 | 0.472 |
| | GLUT4 | 0.0297 ± 0.005 | 0.0385 ± 0.0053 | 0.232 |
| | IRβ | 0.0175 ± 0.0042 | 0.0141 ± 0.0028 | 0.513 |
| 9 Month | GLUT1 | 0.0526 ± 0.015 | 0.0528 ± 0.013 | 0.993 |
| | GLUT4 | 0.0034 ± 0.0004 | 0.0079 ± 0.0018 | 0.047 |
| | IRβ | 0.00031 ± 0.00008 | 0.00025 ± 0.00005 | 0.584 |
| 21 Month | GLUT1 | 0.048 ± 0.009 | 0.059 ± 0.007 | 0.343 |
| | GLUT4 | 0.087 ± 0.041 | 0.105 ± 0.037 | 0.764 |
| | IRβ | 31.324 ± 5.151 | 29.962 ± 4.900 | 0.854 |

Values are starting quantity of gene (in pg) normalized to starting quantity of ribosomal protein s15 (in pg). Values reported as mean ± sem.

Protein data at 9 and 21 month for those metabolic mediators evaluated in renal samples is shown in Table 22, with no differences found between groups at either time point.

**Table 22. Summary of Metabolic Mediators in Adult Renal Samples: Western Immunoblot Results.**

| | | Control | Treatment | p value |
|---|---|---|---|---|
| 9 Month | IRβ | 1.12 ± 0.22 | 0.68 ± 0.21 | 0.188 |
| 21 Month | IRβ | 3.86 ± 1.34 | 2.94 ± 0.72 | 0.588 |

Values are mean Corrected Arbitrary Units ± sem. 50 μg total protein loaded per well. Wells normalized to a common control run on each gel.

**Discussion**

Exposure to excess prenatal androgens has been previously shown to cause growth restriction, alter sexual development and function, and cause the onset of symptoms that closely resemble those seen in women with polycystic ovary syndrome (PCOS). Many women with PCOS display a combination of symptoms collectively known as the metabolic syndrome. In addition to cardiovascular effects, hyperandrogenism, insulin resistance, and impaired glucose tolerance are among the symptoms of this syndrome. Various clinical observations and experimental data exist to support the existence of an interaction between insulin and testosterone (Polderman *et al.* 1994). The exact mechanism of interaction remains debated, but the leading hypothesis appears to be that elevated levels of insulin stimulate the production of androgens. However, it has also been demonstrated that hyperandrogenism can stimulate insulin resistance and reduce glucose tolerance (Holmang *et al.* 1992; Polderman *et al.* 1994).

In rats treated with insulin, Samuelsson *et al.* demonstrated an association between hyperinsulinemia, a common risk factor in PCOS and the metabolic syndrome, and changes in cardiac structure and function (Samuelsson *et al.* 2006). Overall, insulin treatment lead to left ventricular concentric hypertrophy and impaired cardiac function. In insulin-treated rats, myocyte density in the left ventricular wall was significantly reduced while myocyte diameter was significantly increased. The treated rats also had a significantly higher incidence of left ventricular fibrosis (Samuelsson *et al.* 2006). This finding is supported by those of other studies of diabetic humans and animals that have shown cardiac abnormalities, to include myocyte hypertrophy, perivascular fibrosis, and increased quantities of matrix collagen (Taegtmeyer *et al.* 2002; Hardin 1996; Dhalla *et*

*al.* 1998). While not specifically evaluated in the current study, left and right ventricle samples from both the 9 and 21 month adult testosterone treated groups required much more attention during RNA isolation in order to obtain clean samples. This is suggestive of more connective tissue within the ventricles of treated sheep and altered cardiac function.

In the fetal heart, a significant increase in IRβ mRNA concentration was seen in the treatment group at fetal Day 90. As insulin receptors are critical in myocardial growth (Barry *et al.* 2006), this up-regulation of IRβ may be an early indication of fetal adaptation to altered insulin responsiveness. Polderman *et al.* have suggested that testosterone alters insulin signaling by interacting at the insulin receptor level, rather than downstream (Polderman *et al.* 1994). The up-regulation of IRβ at fetal Day 90 may be an attempt to maintain normal insulin signaling by increasing receptor availability in order to offset the competitive binding of testosterone with the insulin receptor. While the significance is gone in the mRNA data by 9 months of age, there was still a significant increase in the IRβ concentration in the left ventricle of the treatment group, suggesting that the cardiac tissues are still trying to compensate. By 21 months of age, there was a reversal between the groups, and IRβ concentration in the left ventricle is significantly reduced in the treatment group. The reversal was also present for mRNA concentration, although not significant. These findings are in agreement with Desrois *et al.* who also reported a decrease in IRβ concentration associated with insulin resistance in type 2 diabetic rat hearts (Desrois *et al.* 2004).

In the sheep model of PCOS, hyperinsulinemia that occurs following exposure to excess fetal androgen may also reflect alterations in feedback mechanisms that regulate

the interaction between angiotensin II (Ang II) signaling via AT1 receptors and insulin signaling via the insulin receptor.

Insulin sensitivity is important in the regulation of glucose uptake and metabolism and has been shown to be altered by prenatal exposure to excess androgens. The timing of the androgen exposure has differing effects on insulin's regulation and action. In female rhesus monkeys exposed to androgen excess during late gestation, insulin action, but not secretion, is impaired. This is in contrast to exposure during early gestation when glucose-mediated β cell secretion of insulin is impaired (Bruns *et al.* 2004; Eisner *et al.* 2000). Recabarren *et al.* have demonstrated hyperinsulinemia during early postnatal life in female offspring from testosterone treated ewes (Recabarren *et al.* 2005). They showed higher basal insulin levels and impaired sensitivity at 5 weeks of age, although no differences remained by 20 weeks of age. There is also evidence from euglycemic insulin clamp studies and insulin sensitivity indices that prenatal testosterone treatment leads to insulin resistance in offspring as old as 18 months of age (Kavoussi *et al.* 2006; Lee JS *et al.* 2006), presumably through the insulin receptor. The hyperinsulinemia in this sheep model may result in increased interaction between insulin and AT1 receptors that subsequently allows cardiac remodeling in the left ventricle. In the current study, the decrease in the 21 month right ventricle AT1 mRNA concentration reported in the previous chapter may be a result of a feedback mechanism in response to an increased interaction between Ang II and the insulin receptor. Ang II, the active component of the renin-angiotensin system (RAS), is important in the regulation of blood pressure. All components of the RAS have been detected in the vasculature and Ang II is known to

play a pathogenic role in the development of cardiac hypertrophy and hypertension (Levy 2005).

The differences in IRβ concentration between the left and right ventricles suggest that different pathways are involved in regulating cardiac function and glucose metabolism as the offspring age. Coupled with changes seen in eNOS at 9 months reported above and GLUT1 and GLUT4 changes discussed below, the differences between gene expression and protein concentrations of glucose metabolic mediators suggests that other cellular mechanisms are working in the prenatally treated offspring to maintain cardiac function within the left ventricle. Taken together, the reversal of IRβ concentrations seen in the left ventricle starting at fetal Day 90 out to 21 months of age suggests that there is altered metabolic regulation occurring and the onset of insulin resistance with age.

In addition to evaluating insulin receptor concentrations, glucose regulation was studied in cardiac and renal tissues. In rats, the administration of testosterone leads to severe insulin resistance due to a combination of effects that disrupt normal insulin action in muscle tissues, including the loss of GLUT4 translocation to the cell membrane, deficient activation of glycogen synthase, and diminished transport of insulin across the capillary (Nilsson *et al.* 1998). While Holmäng *et al.* have demonstrated that testosterone regulation of insulin sensitivity is not mediated by cortisol, free fatty acids, or by estrogens, but by the androgenic action of testosterone itself (Holmang *et al.* 1992), the exact mechanism for how is still not fully understood.

In the fetal heart, GLUT1 is the dominant glucose transporter and is responsible for basal glucose uptake (Ralphe *et al.* 2005; Barry *et al.* 2006). GLUT4 is an insulin-

responsive glucose transporter that translocates to the plasma membrane in response to insulin signaling. Postnatally, cardiac GLUT1 decreases and GLUT4 increases (Wang and Hu 1991; Santalucia *et al.* 1992; Postic *et al.* 1994; Ralphe *et al.* 2005). As the two predominant glucose transporters in cardiac muscle (Barry *et al.* 2006), changes in GLUT1 and GLUT4 transporters may have significant effects on cardiac development in a sub-optimal uterine environment.

In adult human and rat type II diabetes, alterations in the concentration or translocation of GLUT4 have been observed to cause insulin resistance (Thamotharan *et al.* 2005; James *et al.* 1989; Maianu *et al.* 2001; Ryder *et al.* 2000). In studying this condition, Thamotharan *et al.* found that the altered *in utero* environment associated with intrauterine growth restriction was associated with adaptive changes that persisted into adulthood (Thamotharan *et al.* 2005). Among those changes was an increase in the skeletal muscle basal plasma membrane-associated GLUT4 concentrations, despite total GLUT4 content being decreased (Thamotharan *et al.* 2005). They also found that the distribution of GLUT4 was unresponsive to exogenous insulin, either in growth restricted newborns or adult offspring. Ralphe *et al.* found similar changes in GLUT4 localization in the stressed ovine fetal heart, where GLUT4 localized to the sarcolemma membrane where it could contribute to glucose uptake (Ralphe *et al.* 2005). However, unlike Thamotharan *et al.* (Thamotharan *et al.* 2005), they reported an increase in left ventricular GLUT4 protein. This increase, in agreement with what Thamotharan *et al.* reported, was not accompanied by a change in mRNA levels indicating that the regulation must occur post-translationally (Ralphe *et al.* 2005). These findings are in contrast to the findings by Tsirka *et al.* who reported significantly reduced GLUT1 and GLUT4 mRNA

and protein concentrations in adult rats showing intrauterine growth restriction (Tsirka *et al.* 2001).

The results of the current study are in agreement with those of Tsirka *et al.* By 9 months of age, GLUT4 mRNA concentration tended to be reduced in the right ventricle of offspring from the testosterone-treated ewes, and by 21 months of age was significantly reduced in the right ventricle. GLUT1 mRNA concentrations were also significantly reduced in both the left and right ventricles at 21 months of age in the treatment group. Tian *et al.* reported that hearts from cardiac-selective GLUT4 deficiency in mice had irreversible systolic and diastolic dysfunction with mild hypertrophy. This deficiency makes the heart more susceptible to ischemic injury and cardiac GLUT4 down-regulation has been reported in a number of disease states, including diabetes and cardiac hypertrophy (Tian and Abel 2001). Despite a lack of difference in right ventricle weights at any time point in the current study, the decreases seen in the right ventricle GLUT4 mRNA concentrations suggest changes are occurring. Additional studies looking at the lungs and pulmonary circulation are necessary to elucidate what is occurring on the right side of the heart.

By 21 months of age, the altered GLUT1 and GLUT4 mRNA concentrations seen in both the left and right ventricles of the treatment group suggests that the adult heart has become less responsive to insulin-stimulated glucose uptake. This may contribute to increased morbidity from ischemic heart disease that has been reported in low birth-weight offspring (Tsirka *et al.* 2001). A possible cardiac outcome in a hyperinsulinemic and hyperlipidemic environment, such as is present in the metabolic syndrome, is a metabolic maladaption that results in a pathological accumulation of glucose metabolites

and fatty acid metabolites within the cardiomyocyte (Young *et al.* 2002). The result is an inefficient use of energy substrates despite an overabundance of each and results in cardiac pathologies.

When taken as a whole, the data supports the development of insulin resistance as offspring exposed to prenatal androgen excess age. The lack of changes in glucose transporters during early development suggests that there is less of a direct effect of excess androgen on fetal metabolic regulation and more of an indirect effect on other aspects of the uterine environment, such as placental development. However, the increase in cardiac IRβ mRNA concentration at fetal Day 90 suggests a compensatory response by the heart has started. Androgen exposure may have more of a direct effect on other aspects of fetal development, such as in the hypothalamic-pituitary-gonadal axis and the reproductive effects seen with this ovine model of PCOS as previously described by numerous researchers. The lack of many early changes in the metabolic mediators evaluated in this study suggests that the effects seen later in insulin signaling and glucose metabolism may be a result of fetal programming occurring as a result of placental insufficiency. An additional fetal time point around 130 – 140 dGA may have helped detect changes that appear later in fetal development.

# CHAPTER V

## SUMMARY

During fetal development, a complex interaction between numerous growth factors, receptors, and signaling pathways takes place to establish the regulation of the various cardiovascular and metabolic functions necessary for normal development, both during the fetal period as well as later in life. Alterations in the fetal environment during this time can modify normal gene expression, protein concentrations, signaling cascades, and other physiologic functions. This fetal programming is typically a survival adaptation for the developing fetus, but often leads to complications later in adulthood.

The occurrence of fetal growth restriction is a significant health concern worldwide. It leads to infant mortality and morbidity and is a major risk factor for the development of cardiovascular diseases, hypertension, type II diabetes, obesity, and various reproductive disorders. The ovine model of polycystic ovary syndrome (PCOS) leads to fetal growth restriction and has clinical relevance as women with PCOS, in addition to the endocrine effects, also develop signs of the metabolic syndrome and have a higher incidence of fetal growth restricted offspring. The elevated androgens available during fetal development and differentiation act as potent gene transcription factors and induce other transcription factors that can permanently alter gene concentration and reprogram multiple organ systems. However, the molecular aspects of these changes are still not well understood. The characterization of mediators of vascular and metabolic

development in fetal and adult tissues in the current study helps to more thoroughly understand the effects of prenatal androgen excess on fetal development and subsequent adult disease states. By characterizing the protein and mRNA concentration patterns of the vascular and metabolic mediators discussed earlier, a better understanding of the fetal environment and how it influences development and programming will help to treat, and ultimately prevent, adulthood diseases. The effects of such a fetal environment are summarized in Figure 9.

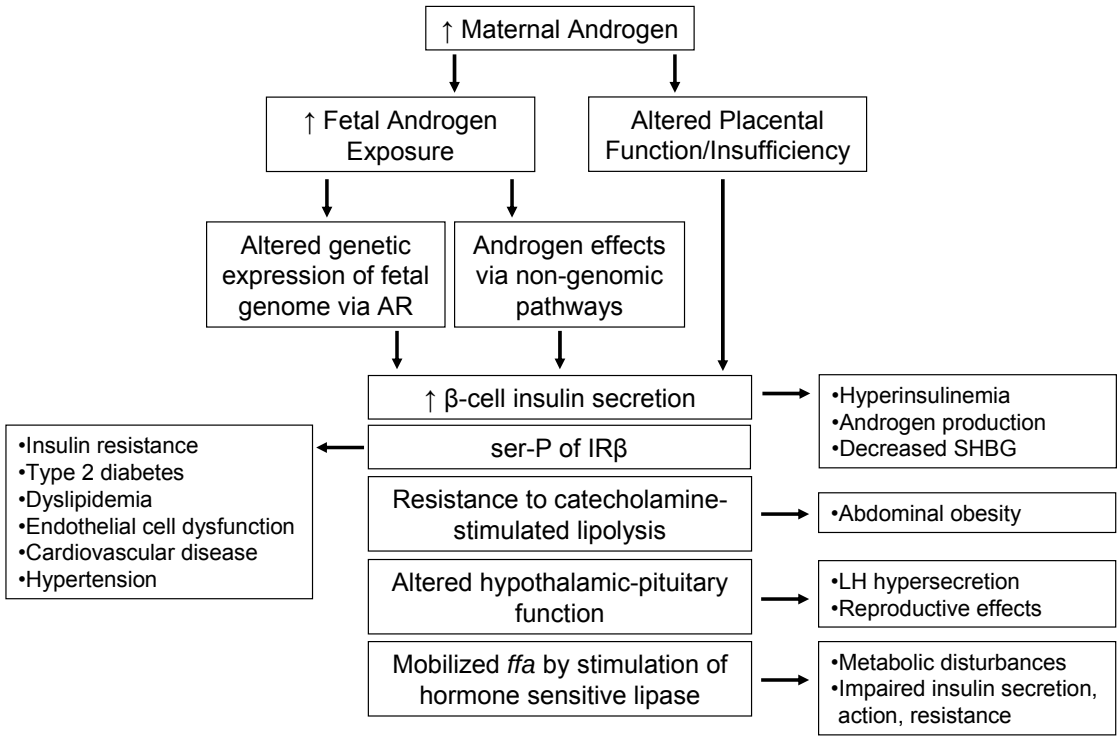


**Figure 9. Summary of effects of fetal androgen exposure.**

**Attachment 1**
**cDNA Sequences**

**Angiopoietin 1 (Ang1)**
PCR Product Size: 259 bp
Accession #: AY881028

***ttgccataaccagtcagag***gcagtacactctaagaattgagttattggactgggaaggaaaccgagcctattcacaatacgaca
gattccacataggaaatgaaaagcagaactacaggttgtatttgaagggtcacactgggacagcaggaaaacagagcagcctg
atcttgcatggtgctgatttcagcactaaagatgctgataatgacaactgcatgtgcaaatgtgccctcatgc***taacaggaggctg***
***gtggtt***

**Angiopoietin 2 (Ang2)**
PCR Product Size: 272 bp
Accession #: AY881029

***gaccgctgtgatgataggaa***atagggaccaacctgctcaatcaaactgcagagcagacccggaagttaacagatgttgaagcc
caagtattaaatcagacaacaagacttgaacttcagcttctagaacactccctttctacaaacaaattggaaaaacagattttggatc
agaccagtgaaataagcaaactgcaagataagaacagcttcctggaaaagaaagtcctagacatggaagacaagcacatagt***t***
***caacttcgggtcaatcaaa***

**Angiotensin Receptor Type 1 (AT1)**
PCR Product Size: 488 bp
Accession #: NM_001009744

***gctgacttatgctttttact***gactttgccgctgtgggctgtctacactgctatggaataccgctggcccttcggcaattacctatgtaag
atcgcttccggcagtgtcagtttcaacctctacgccagtgtgtttctactcacatgtctaagcattgaccgctacctggctattgttcac
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cggtagggctgggcctcaccaagaatatactgggattcttgtttccttttctgatcattcttacaagctacactcttatttggaagaccct
caagaaggcttatgaaatt***cagaagaacaaaccaaggaa***

**Angiotensin Receptor Type 2 (AT2)**
PCR Product Size: 526 bp
Accession #: AF254444

***gtttccaggatttacatctt***caacctagctgtggctgacttacttcttttggctactcttcctctctgggcaacctattattctcatagata
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gtgttgataggtaccaatctgtcatttaccccttctgtctcaaagaagaaatccctggcaagcatcttatatagttccccttggttggt
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tcccacctgagaagtatgcccagtggtcagctgggattgctttaatgaaaaatatccttggttttattatccctttaatattcatagcaa
catgttatttcggaatcagaaaacatctactgaagaccaatagctatgggaa***gaataggataactcgtgacc***

**Endothelial Nitric Oxide Synthase (eNOS)**

PCR Product Size: 391 bp

Accession #: DQ015701

***tgcatgacattgaggagcaaagggc***tgcagcccgcccccatgaccctggtgttcggctgccggtgctcccaactcgaccatctc
taccgcgacgaggtgcaggacgcccaggagcgcggggtgtttggccgcgtcctcaccgccttctcccgggaacctgacagcc
ccaagacctacgtacaggacatcctgagaaccgagctggctgccgaggtgcaccgcgtgctgtgcctcgagcggggccacat
gtttgtctgcggcgacgtcactatggcaaccagcgtcctgcagacggtgcagcgcatcttggcgacagagggcggcatggag
ctggacgaggcgggcgacgtcatcggcgtgctgcggga***tcagcaacgctatcacgaggacat***

**Glucose Transporter 1 (GLUT1)**

PCR Product Size: 380 bp

Accession #: U89029

***taggtgaagccaggatgtg***ggtgaaggagactctggctgataaaaaaataataacaaaatcagtaataaaaaattataaaac
ctgttgcttgtctgaatagatctgagcaacagtcttttgttaaaatcctggagccgttaatgtcctgaatattcttctggacatcactgct
ggctgaaggaaggagccccaggcccgggtctgctgagatctatcagtttgagagtctcatccagctgcctgcgctctgagagat
ccttgggccactgggagcagaccgggctggcggtgtggggcacctcacacttgggaatcagcacccaggggtggaacagc
tcctcaggtgtcttgtcac***tttggcttgctcctccctgtc***

**Glucose Transporter 4 (GLUT4)**

PCR Product Size: 155 bp

Accession #: AY949177

***tggccttctttgaaattggccctg***gccccatcccctggttcatcgtggccgagctcttcagccagggaccccgcccagcggccat
ggcagtggctgggttctccaactggacgtgcaatttcatcattggc***atgggtttccagtatgtggcggat***

**Insulin Receptor Beta (IRβ)**

PCR Product Size: 415 bp

Accession #: AY157728

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tccttcttccggaagttacgtctgattcgaggggagaccttggaaattgggaactactctttctacgccttggacaaccagaaccta
aggcagctatgggactggggcaaacacaacctcaccatcactcaggggaaactcttcttccactataaccccaaactgtgcctgt
cggaaattcacaagatggaggaagtttcaggaaccaaggggcgccaggagagaaatgacatgctctaaagaccaatgggga
ccaggcatcctgtgaaaatgaattacttaaattttcttacattcggacatcttat***gacaagatcttgctgaaatgg***

**Ribosomal Protein s15 (s15)**

PCR Product Size: 134 bp

Accession #: NM_001018

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gattggccactacctaggcgagttctccat***cacctacaagcccgtaaagca***

**RNA Polymerase II (Pol II)**

PCR Product Size: 332 bp

Accession #: CD288457

***agtccaacatgctgacggacatga***ccctgcagggcatagagcagatcagcaaggtgtacatgcacttgccgcagactgacaa
caagaagaagatcatcatcacagaggacggggagttcaaggccctgcaggagtggatcctggagacggacggtgtgagcct
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ttgaggctgtacggaaggccctggagcgggagctgtaccacgtcatctccttcgacggctcc***tacgtcaattaccggcacttgg
ct***

**Tunica Interna Endothelial Cell Kinase-2 (Tie-2)**

PCR Product Size: 524 bp

Accession #: AY881030

***gagacagaacacgaaggact***tgcatccttctggttcactacacctttctttacaagttctgccaaatgtgtgcggttcacacgcctt
ctcacacgttctccccataaacccaggagggcaaatgcattccccagtatcttcatggcaaatcccattgttcatacaagcagtgc
agatgcggttacattcaggtccccacttctgagcttcacatctccggactatgagcctggtgaaggctgaggtgaagaggtttcct
cctatgtacctggccgagtacacgccagcatcctggggctgagcgtgaggcagatgcacttccagaatgtcagggacttcgtgc
cggggcaccgagtggatgaaggaaccatttttgtaaatcactgcatcttcttctttaatcaatacctttttgaaagatatgttcacattat
ctcccctgtccacagtcatagttaaagtagctggtagaaaggaagcttgttggcgcatcttcatggtcc***gtatccttattgcctgtcc
tc***

**Vascular Endothelial Growth Factor (VEGF)**

PCR Product Size: 251 bp

Accession #: AF071015

***ttgccttgctgctctaccttc***accatgccaagtggtcccaggctgcacccatggcagaaggagggcagaaaccccatgaagtga
tgaagttcatggatgtctaccagcgcagcttctgccgtcccattgagaccctggtggacatcttccaggagtacccagatgagatt
gagttcattttcaagccgtcctgtgtgcccctgatgcggtgcgggggctgctgtaatgacg***aaagtctggaagtgtgtgccc***

**Vascular Endothelial Growth Factor Receptor 1 (VEGF R1)**

PCR Product Size: 195 bp

Accession #: AF063657

***tctatagcaccaagagcgacgtgt***ggtcctacggagtgcttctgtgggagatcttctccttaggcgggtccccctacccgggtgt
gcagatggacgaggacttctgcagccgcctgaaggacgggatgaggatgcgggcacccgagtacgccacgcctgaaatcta
ccaga***tcatgttggactgctggcacaaag***

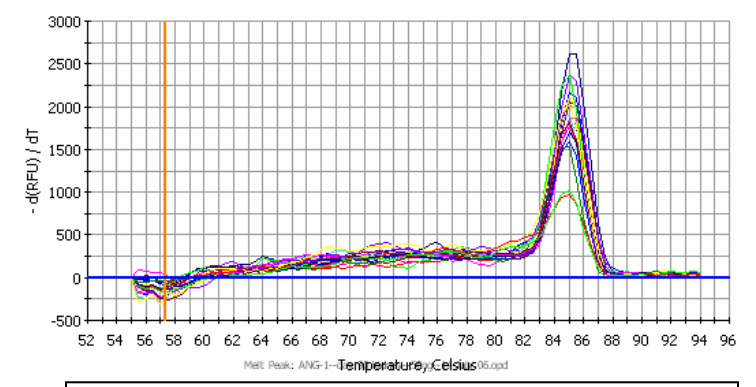
**Vascular Endothelial Growth Factor Receptor 2 (VEGF R2)**

PCR Product Size: 542 bp

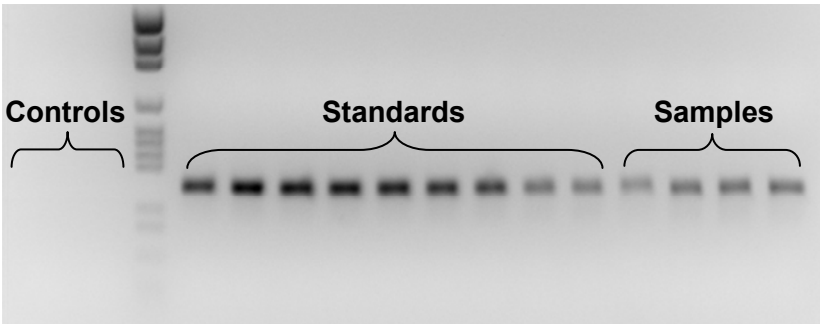
Accession #: XM_050674

***ttccactgggaataccttcttcg***aagcatcagcataaaaaacttataaaccgggacctaaaaacccagtctgggactgaaatga
agaagtttttgagcaccttgactatagatggtgtaacccggagtgaccaggggtggtatatctgtgcagcttccagtgggctgatg
accaagaagaacagcacgttcgtccgggtacatgaaaagccgtttgttgctttcgg***tagtggcatggaatccttggtgga***

**Attachment 2**

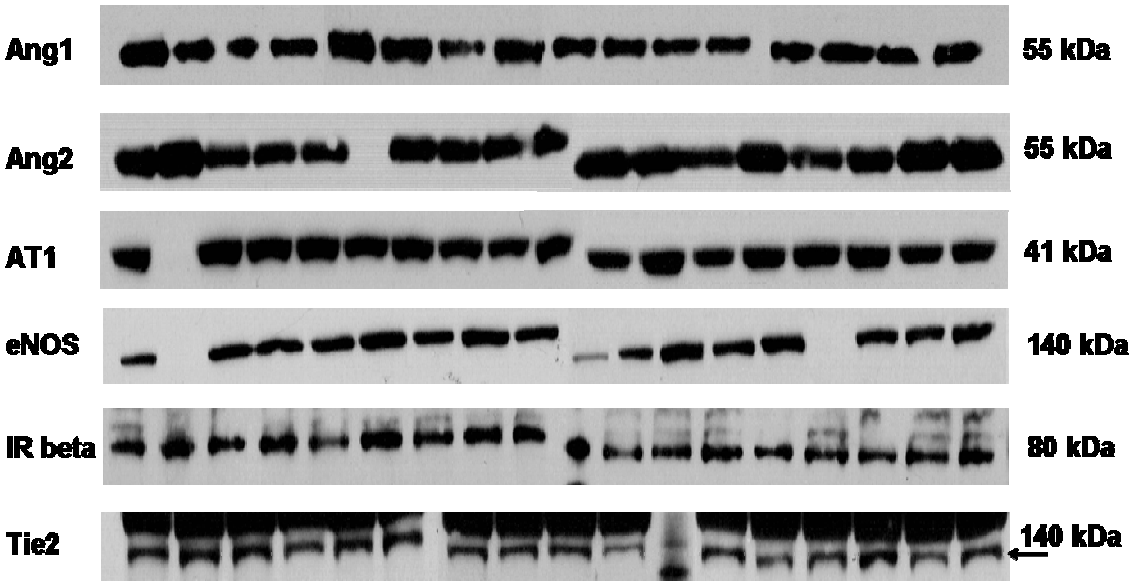



Melt curve from real-time PCR showing all samples peaking at the same temperature.




2% agarose gel showing standards and samples running at correct size and no product in the SYBR and RT controls.

**Attachment 3**




Representative Western immunoblots electrophoresed through NuPAGE 4 – 12% Bis-Tris gels. 50 µg total protein loaded per well.

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